An investigation into the protection of human muscle from hypoxia-reoxygenation injury:

Studies using isolated atrial trabeculae obtained from human right atrial appendage harvested during cardiac surgery

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Declaration

I, Abdul Malik, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Ischaemic heart disease remains the leading cause of death worldwide and its major manifestation is through an acute myocardial infarction (AMI). This usually presents as an ST segment elevation myocardial infarction (STEMI), where an acute plaque rupture leads to a thrombotic occlusion of the coronary artery rendering the myocardium ischaemic that eventually leads to cell death. The most effective strategy of treating this is via percutaneous coronary intervention (PCI) whereby the thrombus is aspirated and stent is implanted within the narrowed segment of the artery. Other treatment options include thrombolysis or emergent coronary artery bypass grafting (CABG) surgery. Although reperfusion is a pre-requisite for myocardial salvage, the process itself is capable of inducing cell death in addition to that caused by myocardial ischaemia - a process termed 'lethal reperfusion injury'. New treatment strategies are required to protect the heart against the detrimental effects of acute ischaemia-reperfusion injury (IRI). Stromal cell-derived factor 1α (SDF-1α or CXCL12), acting through its cognate receptor on target cell membranes has been recognised and demonstrated in animal models to limit myocardial infarction size

following acute ischaemia reperfusion injury. We have already established that SDF-1α is an important humoral factor mediating the effects of remote ischaemic conditioning (RIC) such as reducing infarction size in a rat *in vivo* model as well as improving functional recovery of rat cardiac papillary muscle in an *ex vivo* model. Whether, SDF-1α can protect human heart tissue is not known, and is investigated here using isolated human atrial trabeculae exposed to simulated IRI.

A human atrial trabecular model utilising simulated ischaemia-reperfusion injury was used to reiterate the existence of ischaemic preconditioning in human tissue. The model was characterised using various stabilisation, simulated ischaemia and reperfusion times and the challenges encountered with this model are discussed. Human atrial trabeculae obtained during elective cardiac surgery were suspended in organ baths and superfused with modified Tyrode's solution. Using the optimum stabilisation, simulated ischaemia and reperfusion times, these trabeculae were then subjected to an ischaemic insult. Some of these were preceded by a preconditioning protocol whilst others were pretreated with SDF-1α prior to the simulated ischaemic insult. The end point for the human model was the functional recovery of myocardial contractility. Unlike previous

experiments, the trabeculae were stretched to the peak of the Frank-Starling curve prior to assignment to various protocols. The effect of reduced number of stretches is also compared to multiple stretches.

The results of this study demonstrate that (a) ischaemic preconditioning is effective in a human model despite evolution of advancing medical therapy, (b) contrary to earlier data, I was unable to demonstrate that stretch caused preconditioning of the human myocardium, (c) SDF-1α act as a preconditioning mimetic and protects it from lethal reperfusion injury and (d) the protection appears to be mediated through its cognate CXCR4 receptors and at least partly via activation of intracellular kinases such as extracellular signal-regulated kinases (ERK).

In summary, despite advances in therapy, myocardial infarction is associated with considerable morbidity and mortality. The present study demonstrates the ability of SDF-1α to protect the human atrial tissue and may involve the RISK pathway akin to all forms of conditioning.

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ACEi Angiotensin-Converting Enzyme inhibitor

AMI Acute Myocardial Infarction

ANOVA Analysis of Variance

ANP Atrial Naturetic Peptide

ATP Adenosine Tri-Phosphate

BCA Bicinchoninic Acid

BHF British Heart Foundation

BMS Bare Metal Stent

BSA Bovine Serum Albumin

Ca Calcium

CABG Coronary Artery Bypass Grafting

CAD Coronary Artery Disease

CCG Clinical Commissioning Group

CHD Coronary Heart Disease

CM Cardiomyocytes

CPK Creatine PhosphoKinase

CRT Cardiac Resynchronisation Therapy

CVD Cardiovascular Disease

DAG Diacylglycerol

DES Drug Eluting Stent

ECG Electrocardiogram

ECL Enhanced Chemiluminescence

EDTA Ethylenediaminetetraacetic Acid

eNOS endothelial Nitric Oxide Synthase

ERK Extracellular Regulated Kinase

GPCR G-Protein Coupled Receptor

HSP 72i Heat Shock Protein inducible

ICD Implantable Cardioverter Defibrillator

IRA Infarct Related Artery

IRI Ischaemia Reperfusion Injury

LVEDP Left Ventricular End Diastolic Pressure

MAPK Mitogen-Activated Protein Kinase

MPTP Mitochondrial Permeability Transition Pore

NHS National Health Service

NO Nitric Oxide

NSTEMI Non ST-Elevation Myocardial Infarction

OCT Optimal Cutting Temperature

PBS Phosphate-Buffered Saline

PCI Percutaneous Coronary Intervention

PI 3-K Phosphoinositide 3-Kinase

PKC Protein Kinase C

PKG Protein Kinase G

PLC Phospholipase C

PO_4 Phosphate

PPCI Primary Percutaneous Coronary Intervention

QOF Quality and Outcomes Framework

RIC Remote Ischaemic Conditioning

RISK Reperfusion Injury Salvage Kinase

ROS Reactive Oxygen Species

SDF-1α Stromal-cell Derived Factor 1 alpha

SDS-PAGE Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis

STAT Signal Transducer and Activator of Transcription

STEMI ST-Elevation Myocardial Infarction

SWOP Second Window of Protection

TBS Tris-Buffered Saline

TCFA Thin Capped Fibroatheroma

TEMED Tetramethylethylenediamine

TNFα Tumor Necrosis Factor-alpha

UK United Kingdom

VAD Ventricular Assist Device

WHO World Health Organisation

List of Publications: Posters

Stromal cell derived factor 1 alpha is a mediator of conditioning in human and rat myocardium.

Poster presentation, British Cardiovascular Society, Manchester (June 2015).

The combination of antegrade and retrograde cardioplegia reduces peri-operative myocardial injury in patients undergoing elective aortic valve replacements.

Poster and oral presentation, joint meeting JCTS & Ireland + ACTA, Manchester (March 2015).

Remote ischaemic preconditioning in cardiac surgery: Giving your limbs to protect your heart.

Poster presentation, Institute of Cardiovascular Research, University College London (August 2013).

Myocardial preservation is enhanced by the addition of retrograde cardioplegia in patients undergoing first time coronary artery bypass graft surgery.

Poster and oral presentation, Society for Cardiothoracic Surgeon, Brighton (March 2013).

The protective effects of remote ischaemic preconditioning in patients undergoing elective cardiac surgery.

Poster and oral presentation, Society for Cardiothoracic Surgeon, Brighton (March 2013).

The effects of multi-limb remote ischaemic surgery preconditioning in patients undergoing cardiac bypass.

Poster presentation, British Cardiovascular Society (June 2013).

Remote ischaemic preconditioning in diabetic patients undergoing elective coronary artery bypass graft surgery.

Poster presentation, British Cardiovascular Society (June 2013).

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Malik A, Bromage DI, He Z, Candilio L, Harmaneh A, Tafener S, Davidson SM, Yellon DM. Exogenous SDF-1α protects human myocardium from hypoxia-reoxygenation injury via CXCR4. *Cardiovascular Drugs and Therapy*. 2015; 29(6): 589-592.

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CHAPTER 1: Introduction

1.1 Ischaemic Heart Disease: Magnitude of burden

Data from the Quality and Outcomes Framework (QOF) report that 2.3 million people in the United Kingdom (UK), live with coronary heart disease (CHD). The highest and lowest prevalence being in Scotland and Northern Ireland respectively. £4.3 billion pounds was spent treating cardiovascular disease (CVD) through Clinical Commissioning Groups (CCGs) through the National Health Service (NHS) England between 2012/2013. CVD remains one of the leading causes of mortality and morbidity within the UK as well as globally. In 2014, CVD accounted for 27 percent of all UK deaths while malignancies lead the charts at 29 percent. Though CVD exists in a variety of forms, CHD represents majority of deaths representing almost 45 percent due to CVD. In 2014, 15 percent and 10 percent of male and female deaths were from CHD totaling at 69,000 deaths. Thus, CHD by far is the biggest single cause of death in the UK. In men, CHD also represents the single most common cause of premature death (defined as death before the age of 75 years) accounting for around 16, 800 deaths (15 percent of male premature deaths).

On a global basis, figures from the World Health Organisation (WHO) clearly shows CVD to be the leading cause of death with more than 17.5 million deaths due to CVD in 2012 (31% of all global deaths); 7.4 million deaths were due to CHD despite advances in treatment and overall reduction in mortality over the years (1).

1.2 A historical perspective of coronary heart disease

Angina pectoris is derived from the Latin angina *"infection of the throat"*; Latin pectus *"chest"* (2).

CHD represents a spectrum of disease from angina, unstable angina and myocardial infarction (3). William Heberden first described classical angina pectoris in 1768 before presenting it to the Royal College of Physicians in 1768 prior to its appearance in the Medical Transactions of the College in 1772 (4). Both typical and variant anginas were described that still hold true today. Although Heberden was one of the earlier physicians to describe angina pectoris, there is also a case described in the memoirs of the Earl of Clarendon 1632 (5). During that period of time, pathophysiology was unknown and treatment consisted of bed rest, bloodletting and a tincture of opium, or a combination thereof. Towards the end of the century in 1799,

Caleb H Parry hypothesised that angina pectoris (then referred to as Syncope Anginosa) was due to coronary ossification (calcification) and that it predominated in men aged 50 years and above and rarely occurred in women and children (6). During the 18th and 19th century, medical knowledge focused on clinical observation and clinical dissection before the emergence of Cardiovascular Sciences towards the end of the 19th century and beginning of 20th century where an interplay between science and medicine was sought to better understand the biology of cardiovascular disease.

Following Heberdens first description of angina, it took almost a century for pathologists to describe thrombotic occlusions in addition to coronary ossification in relation to coronary artery disease.

Towards the end of the 19th century, vascular biologist discovered occlusion of the coronary artery in dogs caused "quivering" of the ventricles that was rapidly fatal (7, 8). In 1879 pathologist Ludvig Hektoen concluded coronary thrombosis as the underlying cause for a myocardial infarction (9). In 1910, a pair of Russian physicians trained in pathology described a series of post mortem that resulted from a clinical picture of myocardial infarction (10). Following this James Herrick described total bed rest as a treatment for this

condition (11) before utilising electrocardiography (ECG) to diagnose it (12). This was the standard of care to diagnose and treat this condition until the mid 20th century when further rapid advances were made.

In 1948, the Framingham Heart Study studied the lifestyle of the residents in Massachusetts and published their findings "Factors of Risk in the Development of Coronary Heart Disease" (13). The study was the first to report hypertension and hyperlipidaemia as risk factors for the development of coronary artery disease (CAD). As part of their observation, investigators also noted the development of ischaemic heart disease in women later in life compared to men (13). National programs were subsequently rolled out to educate physicians and the public that led a significant reduction in age-related cardiac deaths.

In 1961 coronary care units were established. Prior to this patients who were fortunate enough to survive a myocardial infarct were admitted anywhere within a hospital and usually as far as possible from the nurses station in order to allow them complete rest. Unfortunately the death rate was as high as 30%, most likely due to some form of arrhythmia. Since the development of coronary care units, inpatient mortality has reduced by half owing to continuous

cardiac monitoring, cardio-pulmonary resuscitation and external defibrillation (14).

Back in 1628, William Harvey set stage for the physiology of cardiovascular system to flourish. In his publication of *De Motu Cordis* in 1628, he described the cardiac function and circulation (15). French physiologist Claude Bernard during the 19th century progressed to measure pressures within cardiac chambers and great vessels (16). This followed suit by Werner Forssman who undertook cardiac catheterisation on himself resulting in the first human cardiac catheterisation (17). Further exploration together with Andre Frederic Cournand and Dickinson W Richards (18) led to all 3 being awarded the Nobel Prize in 1958. Since then cardiac catheterisation developed in 1958. This was combined with left ventriculography to study coronary artery disease that became the gold standard for defining coronary anatomy and a platform for surgical treatment by way of coronary revascularisation (19).

It was the pioneering work of Dotter and Judkin that lead to the field of percutaneous coronary intervention with Andreas Grüntzig (1939-1985) becoming the father of such intervention (20). Grüntzig, who was a German radiologist was taught the angioplasty technique from Dotter following a lecture in Germany and then went on to perform

the first coronary balloon angioplasty on a human being with excellent final angiographic results that remained patent at its 10th anniversary. Subsequently bare metal stents (BMS) evolved followed by drug eluting stents (DES), which are utilised mainly today (21).

By the 1970s, in-hospital mortality had reduced from 30% to around 15% and in the first year following hospital discharge approximately 10% of patients of died from left ventricular failure that resulted from larger index infarctions. It was later discovered, through animal studies, that improving the balance between myocardial oxygen supply and demand could reduce infarct size (22). By 1976, Cardiologists were able to unblock occluded coronary vessels with the use of intravenous infusions of streptokinase, which acted as a thrombolytic agent, which essentially dispersed the clot (23). The Italian Group for the Study of Streptokinase in Myocardial Infarction (GISSI) was the first human clinical trial involving 11,712 patients, demonstrating a reduction in early mortality in the setting of an acute infarction (18% relative risk reduction; 13% to 10.7% 21-day hospital mortality) (24). The reduction was further pronounced with the addition of aspirin to streptokinase as demonstrated in the ISIS II trial (25). Interestingly, survival advantages produced by the combination of fibrinolytic therapy and one month of aspirin started

during an acute myocardial infarction seem to be maintained for at least 10 years. Further reduction in mortality was demonstrated when coronary stenting was combined with potent platelet inhibition and especially when the time between symptom onset and arrival to hospital was minimised (26). During this era, public and professional awareness was enhanced and the GISSI trial also became the paradigm for advancement in cardiovascular therapeutics through controlled clinical trials. Based on prior animal studies of induced myocardial infarction, the SAVE (Survival and Ventricular Enlargement) trial (27) was able to support previous findings (CONSENSUS/SOLVD) that ACE inhibition following an infarct with left ventricular impairment reduced mortality amongst these patients (28, 29). The addition of beta-adrenergic blockers and aldosterone blockers further reduced mortality in these patients. In the face of these advances, large infarcts leading to large scar tissue, lead to severe heart failure, therefore implantation of implantable cardioverter defibrillators (ICDs) (30), cardiac resynchronisation therapy (CRT) (31) and ventricular assist devices (VAD) (32) have improved prognosis for this subset of patients.

1.3 Ischaemia of the myocardium

Ischaemia (Greek *ischein* "to restrain" + *haima* "blood") is the restriction of blood supply to an organ or tissue. It occurs when the blood supply is inadequate to meet the tissues metabolic demands resulting in hypoxia, insufficiency of metabolic substrates and accumulation of metabolic waste that ultimately affect cell survival (33). It may occur in a variety of circumstances (decreased supply or increased demand) but in relation to the myocardial ischaemia, the coronary vessel typically becomes either partially or totally occluded resulting in an imbalance between the supply and demand of oxygen and essential nutrients to the myocardium. Atherosclerotic disease is the most common cause of myocardial ischaemia. It can have both stable and unstable elements during which activated vascular wall inflammation may lead to a myocardial infarction that may go undetected or present for the first time as sudden cardiac death or with severe haemodynamic deterioration (34). Clinical features of ischaemia result from this supply-demand imbalance and patients often present with a variety of symptoms including diffuse discomfort in the chest, shoulders and upper extremities that tend to be associated with autonomic features of nausea, vomiting and diaphoresis. The duration and severity of ischaemia often

determines the clinical manifestation that spans from cardiac angina (less than 20 minutes) to a full thickness myocardial infarction (more than 20 minutes). Maroko and colleagues first identified reperfusion as the most effective approach for salvaging acutely ischaemic myocardium (35), (36). In their animal study, they examined the relationship between epicardial ST segment elevation 15 minutes after coronary artery occlusion and myocardial creatine phosphokinase activity (CPK) and histologic appearance 24 hours later and were able to conclude that myocardial tissue can be salvaged if the infarct related coronary artery was reperfused within a timely manner. Treatment of ischaemia consists of medical therapy to control symptoms in situations where angina remains stable or else patients undergo some form of coronary revascularisation. Currently, this can be achieved either through primary percutaneous coronary intervention (PPCI), thrombolysis where PPCI facilities are unavailable in a timely manner, or surgical revascularisation that is referred to as coronary artery bypass grafting (CABG) – This is often necessary when there is a technical challenging lesion or there is severe disease involving either the left main stem coronary artery or there is involvement of two or more vessels (37).

Although reperfusion largely abolishes ischaemia in the infarct related artery (IRA) territory, it also results in the activation of processes that appear to be deleterious thus making myocardial reperfusion a "double edged sword". Some of these processes include:

- Hastening of the necrotic process of irreversibly injured myocytes
- Cell swelling
- No re-flow phenomena
- Haemorrhagic myocardial infarction
- Calcium-oxygen paradox
- Production of oxygen derived free radical that may damage ischaemic myocytes
- Post ischaemic depression of myocardial function "stunning"

Research into the reduction of myocardial injury at the time of reperfusion has now become the interest of many researchers on a global scale since its first description (38).

1.4 Myocardial Ischaemia Reperfusion Injury

Patients encountering an acute ST segment elevation myocardial infarction are treated with timely reperfusion therapy, namely PPCI or thrombolysis as mentioned earlier in section 1.3.

The concept of restoring blood supply to an ischaemic myocardium and paradoxically causing harm was first postulated by Jennings and colleagues in 1960 and was termed lethal reperfusion injury (39). Since its recognition it has become an integral term when describing myocardial injury following an infarct. The injury results in death of cardiomyocytes that were viable immediately before myocardial reperfusion (40-42).

1.4.1 Clinical manifestation of coronary reperfusion

Reperfusion injury results in a spectrum of clinical entities including reperfusion induced arrhythmias, myocardial stunning, microvascular dysfunction, and lethal reperfusion injury (irreversible cell damage or necrosis). The first two are reversible.

1.4.1.1 Reperfusion induced arrhythmias

The observation that reperfusion induced arrhythmias may occur when coronary blood flow is restored to areas of acute ischaemic myocardium by coronary occlusion was originally made in experimental laboratory in the 19th century by Cohnheim and Von Schulthess-Rechberg (43) and later described in 1935 by Tennant and Wiggers (44). There are various types of arrhythmias described in literature including ventricular tachycardia, ventricular premature complexes and idioventricular rhythm. These usually self terminate or are easily treated (45).

1.4.1.2 Myocardial stunning

Heyndrickx et al in 1975 described myocardial stunning as a prolonged post ischaemic dysfunction of viable tissue salvaged by reperfusion (46). Reperfusion of an ischaemic myocardium results in a prolonged period of contractile dysfunction albeit reversible. The myocardium is referred as 'stunned' and requires a prolonged period of time to gain full functional recovery. This can be seen where there is global ischaemia (such as cardiac arrest or cardiac

surgery) or regional ischaemia (as seen during a myocardial infarction) (47).

1.4.1.3 Microvascular dysfunction

This form of reperfusion injury arises from the detrimental effects of intracellular calcium overload and oxidative stress on the myocardial contractile apparatus (48).

The inability to perfuse a previously ischaemic myocardium was first described in 1966 by Krug et al (49). It results due to a combination of several factors including:

- Capillary damage and impaired vasodilation
- External capillary compression by cardiomyocytes swelling and endothelial cells
- Micro-embolisation of material released from atherosclerotic plaque
- Platelet micro-thrombi
- Release of soluble vasomotor and thrombogenic substances
- Neutrophil plugging (50-53)

It manifests as no or sluggish blood flow, impaired myocardial blush and a characteristic coronary flow velocity profile (54). Interestingly, amongst patients who have undergone emergency coronary intervention, 30-40% have evidence of microvascular obstruction on myocardial contrast echocardiography despite having normal coronary blood flow in the infarct related coronary artery during coronary angiography (55, 56). The presence of this phenomenon is usually indicative of larger infarcts, lower LV ejection fractions, adverse LV remodelling and worse clinical outcomes (57, 58). It remains unclear whether microvascular dysfunction (MVD) is independently responsible for cardiomyocytes death or a marker of severe myocardial ischaemia reperfusion injury. Unfortunately, no effective therapy exists to diminish MVD in patients who have undergone coronary angioplasty.

1.4.1.4 Lethal myocardial ischaemia reperfusion injury

The first description of myocardial reperfusion injury stems from Jennings seminal paper in 1960 that suggested the process of restoring blood supply to an ischaemic myocardium can paradoxically induce injury thus attenuating the benefits of

myocardial reperfusion. They reported histological features of reperfused canine ischaemic myocardium that included cell swelling, myofibril contracture, disruption of sarcolemma and the appearance of calcium phosphate particles within the mitochondria (39).

During this process, there is death of cardiomyocytes that were viable immediately prior to reperfusion. This phenomenon partly explains why despite fully restoring blood in an infarct related artery (IRA) in a timely fashion using the latest stents, antiplatelet and antithrombotic therapy, death rate can still be as high as 10% and the heart failure incidence follow an acute infarct can be as high as 25%.

Reperfusion injury has been the subject of a wide range of experimental studies to explore the mechanisms implicated in its pathogenesis; infarct models in animals suggest that this phenomenon may be responsible for up to 50% of the final infarct size and within these experimental models a number of approaches have demonstrated a degree of protection from lethal reperfusion injury (40). Unfortunately these strategies have poorly translated from bench to clinical practice to be applied to real patients. Therefore, it is clear that further laboratory and clinical studies are warranted to further explore reperfusion injury so that these new developments can potentially improve the prognosis following a

myocardial infarction and reduce the risk of developing heart failure at a later stage. The degree of myocardial injury resulting from reperfusion injury in humans, has yet to be quantified.

Important experimental evaluations have concluded key factors that mediate the effects of ischaemia reperfusion injury (IRI), which are discussed below.

1.4.2 Mediators of ischaemia reperfusion injury

1.4.2.1 Oxidative stress (oxygen paradox)

Restoration of blood flow to a previously ischaemic myocardium leads to production of reactive oxygen species (ROS) by a variety of sources (endothelial cells, neutrophils, electron transport chain) that cause cardiomyocyte injury and death through a variety of mechanism including but not limited to direct DNA damage, cell membrane interference and denaturing of enzymes.

1.4.2.2 Calcium overload

Intracellular calcium overload occurs during ischaemia by mechanisms described earlier. At the onset of reperfusion, calcium

overload is further amplified through ROS-mediated injury to sarcoplasmic reticulum and correction of mitochondrial membrane potential (mitochondrial re-energisation). The latter permits regaining of the mitochondrial membrane potential enabling calcium to enter into mitochondria through specific mitochondrial uniporters that eventually cause opening of the mitochondrial permeability transition pore (MPTP).

Opening of the MPTP results in the mitochondrial membrane becoming permeable to molecules less than 1.5 kDa. This promotes swelling of the matrix and disruption to mitochondria. The uncoupling of oxidative phosphorylation leads to a reduction in ATP, without which a cell may not undergo repair following an ischaemic encounter. Oxidative stress along with elevated phosphate elevation and depletion of adenine nucleotide all increase sensitivity of MPTP to calcium. Opening of this pore therefore culminates in necrotic cell death during reperfusion (59).

1.4.2.3 Rapid restoration of physiological pH (pH paradox)

Rapid restoration of pH from less than 7.0 back to within normal physiological range (through washing out of lactic acid and the

triggering of Na/H exchanger & Na/HCO3- symporter) contributes to cell injury during reperfusion by enabling the opening of MPTP and allowing hypercontracture of cardiomyocytes (60).

1.4.2.4 Mitochondrial permeability transition pore (MPTP) opening

The MPTP remains a very important therapeutic target with respect to reperfusion injury. The MPTP is a non-selective channel found in the inner mitochondrial membrane. When it opens, cell death ensues due to mitochondrial depolarisation, uncoupling of oxidative phosphorylation culminating in the depletion of ATP (61, 62). It is an important promoter of cell death, as many mediators of reperfusion injury appear to act on it. The accumulation of lactic acid during anaerobic respiration acts to protect cardiomyocytes during acute ischaemia through inhibition of the MPTP opening and hypercontracture of the cell. During reperfusion, MPTP opens as a result of calcium overload, oxidative stress and rapid pH correction (63). Inhibition of this pore has generated a tremendous research interest and as such, experimental studies have reported a significant reduction of infarct sizes in both animal and human models of

simulated ischaemia-reperfusion injury models using agents such as cyclosporine A (64) which is otherwise known for its common immunosuppressant property in clinical practice.

1.4.3 Coronary atherosclerosis

The principle underlying process responsible for coronary heart disease is coronary atherosclerosis that develops and progresses decades prior to the index event of an acute myocardial infarction or angina. It is an inflammatory process involving the most inner lining of the blood vessel (intima) that is accelerated by proven risk factors (or a combination of) including hypertension, hyperlipidaemia, diabetes mellitus, smoking and genetics (family history) (13). It is composed of cholesterol, smooth muscle and inflammatory cells together with cellular debris. In certain plaques, a lipid core forms under a fibrous cap (composed of smooth muscle cells, elastin and collagen) which itself is covered by endothelium on its luminal aspect. Thin-capped fibroatheromas (TCFA) develop from these plaques when inflammatory cells derived from foam cells originating from circulating monocytes penetrate the arterial wall that weaken and thin out the fibrous cap. If the thin cap of these TCFAs tear, the

thrombogenic lipid core becomes exposed to blood that then results in an intraluminal coronary thrombosis. This may then either

- Spontaneously lyse;
- Remain and become integrated into the arterial wall to further narrow the lumen;
- Progress to total or near total coronary vessel occlusion resulting in an acute coronary event.

There are several factors that determine how the ruptured TCFA behaves including the actual volume and composition of the plaque, degree of luminal narrowing, cap tear size and thrombotic milieu (prothrombotic nature of the blood). Interestingly, it has been demonstrated in patients with sudden cardiac death that repeated cycles of asymptomatic coronary thrombosis occur, which become integrated into the vessel wall prior to the fatal event (65).

An alternative aetiology for coronary thrombosis is when plaque erosion occurs. This is when a thrombus forms on a defect within the endothelial layer that covers the plaque. The plaque itself is not thinned like TCFAs and may not necessarily be inflamed. This type of coronary thrombosis tends to be prevalent in smokers due to the prothrombotic milieu nature of smoking.

The gradual process of atherosclerosis may become accelerated by plaque haemorrhage. This occurs when thin blood vessels with poor integrity grow from the vasa vasorum situated in the most outer layer (adventitia) of blood vessels into the more inner layers and rupture leading to enlargement of plaque (whilst trying to meet the increasing demand of the thickening inner blood vessel). This, however, does not necessarily lead to obliteration of the vessel lumen. In 1987, Seymour Glagov published his seminal article describing the phenomenon of vascular modelling which now bears its name (66). In the Glagov's phenomenon, arteries are thought to alter their size and shape in relation to plaque burden. This positive remodelling essentially maintains the vessels luminal orifice thereby maintaining blood flow to the area it supplies despite an increase in plaque burden. This relationship between plaque burden and luminal orifice has been shown to be maintained with plaque burden up to 45 percent. Thereafter lumen size becomes affected (negative remodelling), due its inability to positively remodel through expansion to accommodate the increasing plaque burden (67).

Following this, as the disease evolves, the luminal diameter of the coronary artery becomes progressively narrowed to varying degrees until up to the point where the patient perceives angina.

Interestingly, atherosclerosis tends to form particularly in the proximal segments of main coronary vessels especially at the point of arterial bifurcation where blood flow haemodynamics becomes altered (68). This slow progression of atherosclerosis may be interrupted by rapid progression related to either asymptomatic plaque disruption resulting in the formation of a non-occlusive intraluminal thrombus or may result in plaque haemorrhage.

1.4.3.1 What happens during an acute myocardial infarction?

The formation of thrombus eventually results in symptomatic coronary occlusion. Plaque rupture is responsible between 66-75% of fatal myocardial infarctions based on post-mortem evaluation. In patients presenting acutely for primary coronary intervention, up to 84% have total coronary occlusion with the remainder having a near total occlusion where flow to distal vessel remains to a limited degree. Quite often, the thrombus extracted during a primary procedure is organised indicating its formation prior to the development of angina.

From a clinical perspective, an acute myocardial infarction is either a non ST segment elevation myocardial infarction (NSTEMI) or a ST

segment elevation myocardial infarction (STEMI). The former is three times more frequent, has a lower incidence of total coronary occlusion and coronary angiography often demonstrates a significant flow-limiting lesion in the order equal to or greater than 70% luminal narrowing. Conversely, STEMI patients frequently have totally occluded coronary vessels with greater myocardial necrotic risks in comparison. There is also a higher degree of plaque rupture seen in STEMIs when compared to NSTEMIs where plaque erosion seems to predominate when coronary vessels are evaluated during the acute presentation using optical computed tomography.

1.4.3.2 Cellular pathophysiology of myocardial ischaemic injury

The coronary vessel that supplies a particular territory determines the area at risk of myocardial ischaemia. During an acute coronary occlusion, prolonged ischaemia (more than 20 minutes) leads to a "wave front" of cell death that begins in the subendocardium, which then expands transmurally to involve the epicardium (69).

Deprivation of nutrients and oxygen leads to a cascade of reactions that culminates in rapid metabolic and biochemical changes within

the cardiomyocytes; oxidative phosphorylation comes to a standstill leading to mitochondrial membrane depolarisation, ATP depletion and inhibition of myocardial contractile function.

Cell metabolism switches from aerobic to anaerobic glycolysis resulting in increased production of lactic acid that translates to a reduction in both intracellular and extracellular pH making the environment more acidic. To compensate for this anomaly, the Na^+/H^+ exchanger brings Na^+ into the cell while extruding H^+. The $3Na^+/2K^+$/ATPase also ceases to function during ischaemia when ATP generation is affected. The ensuing intracellular Na^+ overload (normally the major extracellular cation) triggers the $2Na^+/Ca^{++}$ to function in reverse mode to carry Ca^{++} into the cell whilst extruding Na^+ out of the cell leading to intracellular calcium overload. With continuing ischaemia, waste metabolites accumulate instigating to an increase in osmolality of the intracellular environment leading to a flow of water along this gradient causing cellular swelling. Thus, the net consequence is that of an intracellular milieu which is acidotic, with elevated Ca^{++} and is swollen from the inflow of water (70). The initial acidotic environment generated as a result of ischaemia also inhibits the opening of the mitochondrial permeability transition

pore (MPTP) and prevents cardiomyocytes contraction during this time.

As mentioned earlier the contractile function of cardiomyocytes becomes affected during ischaemia when ATP synthesis comes to a halt as oxidative phosphorylation ceases leading to mitochondrial membrane depolarisation. In an attempt to maintain the mitochondrial membrane potential, the F1F0 ATPase works in reverse mode using energy derived from hydrolysis of ATP leading to an increase in mitochondrial inorganic phosphates. The result is further depletion of ATP and hastening of myocardial contractile function (71).

Reperfusion causes further damage. The pathological mechanism is described below.

1.4.3.3 Cellular pathophysiology of myocardial reperfusion injury

When vessel patency is re-established in the infarct related coronary artery, the changes seen are spectacular; the $3Na^+/2K^+$/ATPase becomes reactivated to actively pump Na^+ into the extracellular space with the return of oxygen and glucose. The Na^+ gradient that is

established across the cell membrane permits the Na^+/Ca^{++} exchanger to work in normal mode (forward direction) thereby extruding Ca^{++} out and bridging Na^+ into cells. In cells subjected to prolonged ischaemia, however the $3Na^+/2K^+$/ATPase is unable to reactivate leading to persistent high intracellular Na^+ concentrations. In such cases, the Na^+/Ca^{++} pump continues to function in reverse mode to exchange intracellular Na^+ for extracellular Ca^{++}. With that in mind, the return of oxidative phosphorylation and generation of free ATP in an environment rich in cytosolic Ca^{++} will cause cardiomyocytes hypercontracture (72).

Moreover, reperfusion leads to a rapid correction of the extracellular acidic pH as excess H^+ are removed - that encourages the Na^+/H^+ exchanger to extrude H^+ for Na^+. This build up of intracellular Na^+ causes the Na^+/Ca^{++} exchanger to work in reverse mode to further increase intracellular Ca^{++} (72). Eventually this process will further augment hypercontracture of the cardiomyocytes as pH normalises. It is thought that the initial low pH environment of ischaemic cardiomyocytes acts as a protective mechanism to inhibit muscle contraction; this is abolished when the pH is normalised at the onset of reperfusion when ATP becomes readily available to endorse hyper contracture in the presence of high calcium concentration (72). In

addition to the electrolyte imbalance, the accumulated waste metabolites are eliminated from the extracellular environment by the return of fluid flow, creating an osmotic gradient, which, in addition to the high cytosolic calcium environment cause cell swelling from water moving along the osmotic gradient.

Within the mitochondria, reperfusion reactivates oxidative phosphorylation leading to the generation of reactive oxygen species, ROS (such as superoxides and hydrogen peroxide) (71). It is through these reactive species that reperfusion injures the myocardium by inducing the opening of mitochondrial permeability transition pores. The endothelial cells and neutrophils also provide a source of ROS including xanthine oxidase and NAPH oxidase respectively. ROS mediates myocardial reperfusion injury by:

- Promoting opening of mitochondrial permeability transition pores
- Causing dysfunction of the sarcoplasmic reticulum
- Attracting neutrophils (chemoattractant)

This, in return, results in:

- Degradation of cell membrane lipids through oxidation (lipid peroxidation)
- Denaturation of enzymes

- Direct DNA damage through oxidation

All this contributes to the deleterious effects of calcium overload.

Physiological restoration of the pH through reactivation of Na^+/H^+ exchanger and washout of lactic acid leads to reversal of MPTP closure and inhibition of contracture. During reperfusion, the mitochondrial potential is also restored thus calcium is driven into the mitochondria which itself trigger off MPTP opening. Within several hours after the onset of reperfusion, neutrophils accumulate within the infarcted myocardium in response to the chemoattractant nature of ROS, activated compliment system and cytokine release.

There is a huge reward to be gained through understanding the mechanism underpinning myocardial ischaemia reperfusion injury and applying this knowledge in treating patients presenting with a ST segment elevation myocardial infarction amongst other disease processes concerning reperfusion injury. Besides timely and effective reperfusion therapy, another important aspect would be to abolish, or at least minimise the detrimental effects of ischaemia reperfusion. In this regard, the work by Murry et al provided a new

conceivable tactic to limit infarct size and introduced the era of ischaemic preconditioning.

1.5 Preconditioning with ischaemia

Definition

"A *brief* period of ischaemia followed by reperfusion renders the heart more resistant to injury from a subsequent longer ischaemic insult rather than accentuating the injury" (73)

This experimental technique essentially produces resistance to the loss of blood supply, and thus oxygen, to tissues of many types.

1.5.1 First description of ischaemic preconditioning

A major breakthrough in the ischaemia-reperfusion story was the work carried out by Murry, Jennings and Reimer on ischaemic preconditioning. This was first published in their seminal paper titled "*Preconditioning with ischaemia: a delay of lethal cell injury in ischemic myocardium*" (74). Their studies were based on their previous experimental work where they had demonstrated that a brief episode of ischaemia reduced the rate at which ATP was

depleted during subsequent ischaemic episodes. By interspersing the ischaemic episodes with normal perfusion, they felt that the myocardium gained benefit by the process of catabolite washing that would have accumulated during ischaemia.

In their landmark experiment, they hypothesised that several brief ischaemic episodes may protect the heart from the deleterious effects of a myocardial infarction and thus reduce infarct size. Indeed, they were able to demonstrate this in canine hearts. In an open chest model, the control group of five dogs were subjected to 40 minutes of circumflex vessel occlusion while in the intervention group, the 7 dogs were subjected to four cycles of 5 minutes circumflex vessel occlusion interrupted by 5 minutes of reperfusion prior to receiving 40 minutes of uninterrupted circumflex occlusion. All groups were then allowed 4 days of reperfusion before histological analysis of area at risk took place. They reported that preconditioning with simulated ischaemia (now termed ischaemic preconditioning) paradoxically reduced infarct size by 75% of that seen in the control groups (see figure 1 below) (72). Moreover they found that the 40 minutes of simulated ischaemia lead to a severe depletion of ATP and cell death in the control group whereas in the preconditioned group, the same level of ATP depletion occurred except that the end result

did not culminate in cell death. Interestingly, the authors also observed during their experiments that during successive application of 5 minutes ischaemia, ST segment changes were less dramatic when compared to the initial occlusion. This was independent of collateral blood flow.

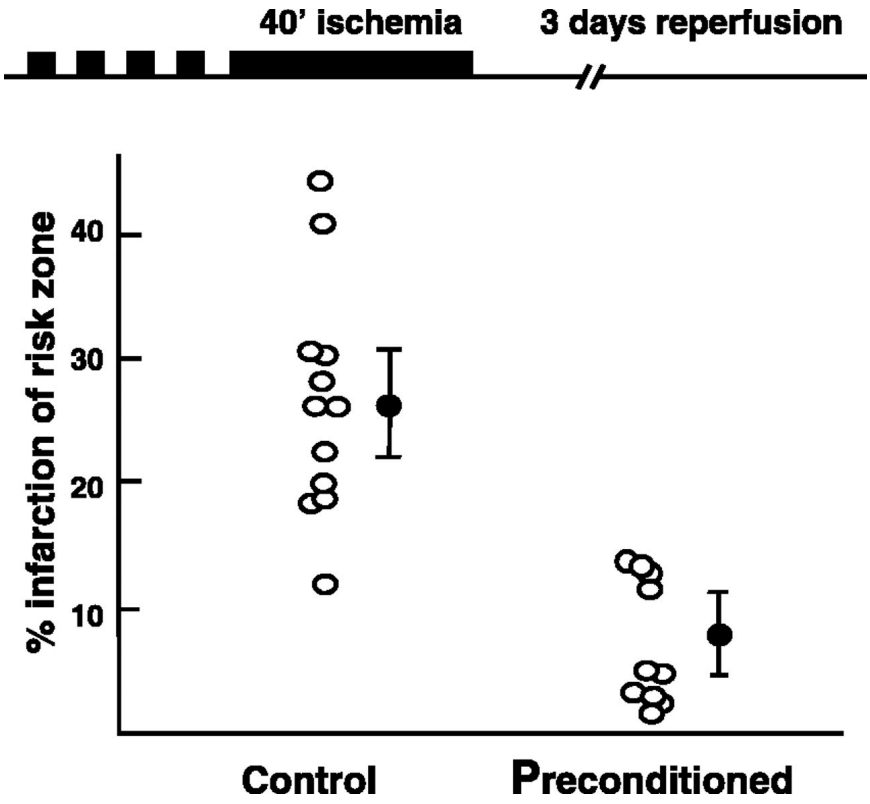


Figure 1.1. Reduction in infarction size with ischaemic preconditioning. Adapted from Murry et al (74).

As important as it may seem, application of such intervention into clinical practice would prove extremely difficult owing to the fact that predicting the onset of myocardial infarction is impossible. However, it can be hypothesised from this study that patients who experience anginal episodes prior to their index myocardial infarction will have a greater degree of myocardial salvage through reperfusion therapy when compared to patients who experience a sudden onset myocardial infarction. This is discussed in section 1.5.8 ahead.

Since the publication of this work, the application of preconditioning has now been extended to demonstrate limited infarct size in the hearts of rabbits (75), rats (76), pigs (77) and other organs to include the skeletal muscle, brain and kidneys (78). Furthermore huge interest has been geared towards underpinning the mechanism of such an innovative intervention, which is discussed later in section 1.5.7. At a later date (1993) Przyklenk describes how preconditioning one myocardial territory confers protection to an area of remote virgin myocardium – subsequently referred to as remote preconditioning (79).

1.5.2 Temporal limitations of ischaemic preconditioning

In the original study conducted by Murry et al (74), a second study was carried out using the identical preconditioning protocol (4x5 min coronary occlusion interspersed with 5 min reperfusion) with the exception that the sustained coronary occlusion was 3 hours rather than 40 minutes (72). In this second group there was no difference observed between the infarct size in the preconditioned and control groups proving the concept that preconditioning acted to delay cell death. However, there is a limitation as to how much ischaemia cells may withstand before irreversible injury occurs leading to cell death. Nao et al (80) demonstrated this in a similar canine model to Murry where the protection conferred by preconditioning was maintained with 60 minutes of coronary occlusion but lost with 90 minutes of occlusion.

Following the pivotal study by Murry et al, and confirmatory studies, experiments were aimed at manipulating the varying components of the preconditioning protocol (now referred to as classical preconditioning) – specifically, the brief antecedent preconditioning stimulus, the duration of intervening reperfusion between the brief and sustained ischaemia and the duration of the subsequent

prolonged index (test) ischaemia in an attempt to assess their individual significance in attaining cardioprotection (81).

1.5.3 Preconditioning stimulus

Original studies employed numerous cycles of ischaemic preconditioning prior to the index ischaemic event to demonstrate cardioprotection; it has since been demonstrated even a single preconditioning stimulus with 90 seconds ischaemia may lower the threshold to induce cardioprotection (82). At the other extreme, if the duration of the preconditioning stimulus itself is large enough to induce cell death, then it is needless to say that this will not confer cardiac protection and in fact may increase infarct size when compared to time-matched controls. Similarly, increasing the cycles of the stimulus mat not necessarily enhance the stimulus and may even result in a loss of efficacy.

Evidence from dog and rabbit models shows that even a small stimulus such as 1.5 mins to 2.5 mins provides an effective preconditioning stimulus (83). In other examples, even a short period of coronary stenosis (15 mins) and transient imbalance of myocardial oxygen supply/demand (such as rapid pace induction)

have been shown to protect rabbit and dog heart from subsequent prolonged ischaemia proving that complete coronary occlusion is not necessary to induce cardioprotection.

Further studies, with a few exceptions, have also concluded that repeated bouts of preconditioning ischaemia do not necessarily yield a cumulative "dose dependent" effect; Li et al (84), for example, found no additional benefit with 6 or 12 bouts of preconditioning ischaemia when controlled with a group of dogs who received a single preconditioning stimulus. In fact, there may even be a loss in efficacy of the preconditioning stimulus with repeated cycles of antecedent ischaemia. This was certainly the case when Cohen subjected conscious rabbits to 40-65, 5 minute occlusions over 3-4 days prior to the index ischaemia and found no protection (85). Interestingly, rabbits may even lose benefit from the preconditioning stimulus in as few as 6-8 repeated bouts of antecedent ischaemia. However, this resistance to protection can be reversed. Cohen et al, discovered that if these repeated bouts of antecedent ischaemic were followed by a rest period and then subjected to a further bout of brief ischaemia, the cardioprotection would be re-established (81).

1.5.4 Time interval between preconditioning stimulus and sustained ischaemia

In the usual or 'typical' preconditioning protocol, the index ischaemia to the tissue or organ is delivered within a short time interval of the index ischaemia. By manipulating this variable, investigators have discovered an association between this time interval and the effectiveness of infarct size reduction. If blood flow is not established between the preconditioning stimulus and index ischaemic occlusion, then it is obvious to say that this would simply result in a greater ischaemic burden. Paradoxically, in experimental models of subtotal coronary occlusions or stenosis, authors have suggested that reperfusion is not a prerequisite to achieve cardioprotection (86). Contrary to this, if the intervening reperfusion is prolonged then there is a significant reduction in benefit that is achieved from this unless a second stimulus is delivered prior to the index ischaemia or if the duration is further increased to 24-72 hours to activate the so-called "second window of protection" (see figure 2 below).

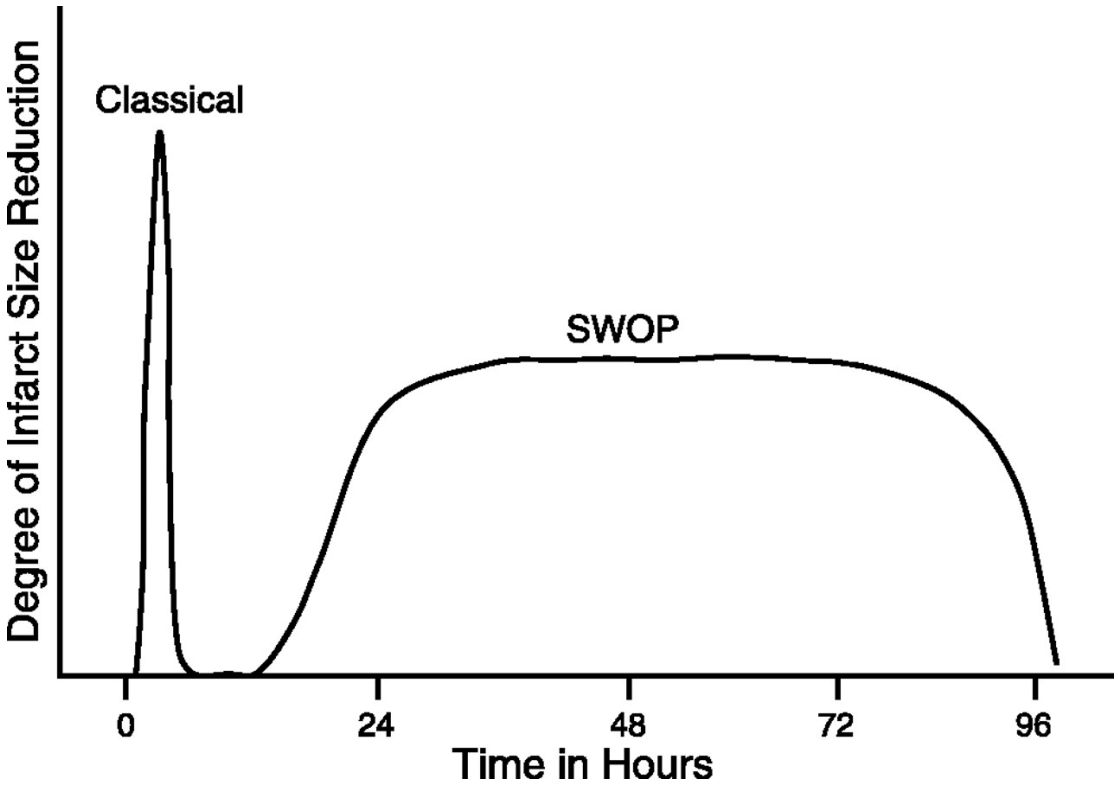


Figure 1.2. Second window of protection adapted from Marber et al (82). In classical preconditioning, the index hypoxia is delivered within 1-2 hours of the preconditioning stimulus to produce a shorter duration but greater efficacy of protection compared to the second window of protection when the delivery of the index hypoxia is delayed at least 24 hours but lasts longer.

The protection achieved immediately following reperfusion is termed early or classical preconditioning. This lasts between 1-2 hours and is lost between 2-24 hours. It was in 1993 that Marber et al (87) and

Kuzuya (88) et al discovered this second "enhanced resistance to infarction" whilst studying with rabbits and dogs.

Marber assessed the role of heat shock protein (HSP) in relation to myocardial salvage; in his experiments he subjected rabbits to either ischaemic preconditioning (four 5 minute coronary ligation interspersed with 10 minute reperfusion) or thermal pretreatment with body temperature elevation to 42^{o}C for 15 minutes, each with corresponding controls. Twenty-four hours later the rabbits hearts were subjected to a period of coronary occlusion for thirty minutes and reperfused for 120 minutes before being euthanised and their hearts assessed for HSP estimation or infarct size. HSP assessed by Western blotting confirmed elevation in both preconditioned and heat treated (with preferential elevation in the former group of rabbits) when comparison was made to their respective controls. Likewise, infarct size, as a percentage of volume at risk, was reduced in both intervention groups. The authors were able to conclude the association of heat shock protein induced by thermal or sublethal ischaemic injury was associated with myocardial salvage (89).

Kuzuya et al confirmed similar findings in an open chest canine model. In their group of dogs, the left anterior descending artery was subjected to 90 minutes of occlusion followed by 5 hours of

reperfusion either immediately or 24 hours following treatment with ischaemic preconditioning (4 cycles of 5 minutes sublethal ischaemia). The sizes of infarct were smaller and similar in both groups when compared to their time-matched controls. No difference in infarct size between control and intervention group was observed when the index infarct was applied 3 or 12 hours following the preconditioning stimulus (90).

It should be noted that although the second window of protection is significant and present universally, it is not as potent as early or classical preconditioning (91). This is demonstrated in figure 2 previously.

The second window of protection is now thought to arise from the upregulation of the 72-kDA inducible heat shock protein (hsp72i), increased transcription or functional upregulation of antioxidant enzyme systems (superoxide dismutase, catalase and glutathione peroxidase), increase cyclooxygenase-2 expression (with concomitant rise in prostaglandins) in addition to nitric oxide synthase (iNOS) amongst activation of other transcription factors (92). It is thought that the particular time delay was obligatory based upon the prerequisite that de novo stress protein synthesis would require a specific time period in become fully expressed.

1.5.5 Duration of index ischaemia

Preconditioning itself does not prevent cardiomyocyte cell necrosis; it serves to delay cell death as evident in Murrys original study where a sustained ischaemic insult for 3 hours abolished the cardioprotective benefits achieved from antecedent ischaemia that was seen during a shorter occlusion of 40 minutes (72). Studies have since proved the efficacy of preconditioning to uphold where the insult lasts anywhere between 30 to 90 minutes but not when this is extended to 3 hours. Ultimately, if the preconditioning intervention is not combined with timely reperfusion, then the protection is lost.

1.5.6 Other aspects of preconditioning

Ischaemic preconditioning not only protects against reperfusion injury but also protects against other detrimental effects of ischaemia including arrhythmias and post ischaemia contractile dysfunction. Animal models have shown a reduction in reperfusion arrhythmias in rats, canine and porcine models of ischaemia-reperfusion. Unlike the second window of protection, ischaemic preconditioning does not appear to alter the bell shaped relationship between the antecedent duration of ischaemia and severity of the arrhythmia. This may

suggest that two different mechanistic processes exist for arrhythmia and size of infarction.

Cohen's group first demonstrated that preconditioning protects against contractile impairment in a rabbit model of ischaemia reperfusion (85) and this was shortly followed by the work of Przyklenk who was able to demonstrate improved systolic improvement in a model of regional ischaemia (79). Furthermore, Caves group were able to demonstrate similar findings in a rat model of global ischaemia where enhanced functional recovery was seen in association with a reduction in enzyme leakage, all suggesting that preconditioning served to reduce necrosis (93).

1.5.7 Mechanism of preconditioning

With all this in mind, the next step was to determine the mechanism of preconditioning. In an attempt to understand the processes involved in preconditioning, investigators have now spent three decades pursuing this subject. The exact mechanism by which preconditioning takes effect remains yet to be fully elucidated particularly its memory aspect; some possible mechanisms have been suggested. What we do know is despite variability with regards

to the duration of preconditioning stimulus and the lasting effect of this, the actual intracellular mechanism is probably similar in all species. Many papers have proposed an explanation through triggers, mediators and effectors.

1.5.7.1 Trigger pathway

The most widely accepted theory for the mechanism is that ischaemia provokes the release of endogenous ligands or *autocoids* that trigger protection by binding to their respective cardiac myocytes cell surface receptors including G protein-coupled and growth factor receptors. This mediates a complex signalling cascade that eventually converge onto one or more end effectors to confer protection (92).

Several ligands have been implicated in this matter including adenosine, bradykinin and opiates that are thought to be released following a brief ischaemic period (92).

GPCR: Adenosine

Adenosine, for example, is a purine nucleoside that is produced from the metabolism of adenosine triphosphate (ATP). During stressful situations (such as antecedent ischaemia), ATP utilisation increases resulting in higher levels of adenosine, which then acts via adenosine A1 receptors to mediate protection. The adenosine receptors are G-protein coupled receptors (GPCR) with 4 subtypes - A1, A2a, A2b, and A3. They have all been shown to mediate cardioprotection (94, 95). Activation of both A1 and A3 receptors prior to lethal ischaemia has been demonstrated in several experimental models to attenuate ischaemia reperfusion injury. Adenosine receptor activation reduces cell death through the activation of mitochondrial ATP dependent K channel (K_{ATP}) as well as through mitogen-activated protein kinases (MAPK) and protein kinase C (PKC) (96) – These are discussed ahead.

To support this, Liu et al (97), investigated this in rabbits. They were able to demonstrate that a 5-minute intracoronary infusion of an adenosine receptor agonist afforded the same degree of cardioprotection as seen in rabbits subjected to a protective preconditioning protocol. Furthermore, they were also able to demonstrate that 2 non-specific adenosine receptor blockers were

able to antagonise the protection mediated through ischaemic preconditioning (97).

GPCR: Opioid

Schultz and colleagues (98) worked with male Wistar rats using naloxone, a non-selective opioid receptor antagonist, to demonstrate the role of opioid receptors in the involvement of ischaemic preconditioning. In their seminal paper, pre-treating the rats with naloxone prior to or immediately after a preconditioning protocol abolished the protective effects of preconditioning (98) seen with opioids, which are now thought to act via kappa and delta opioid receptors (99).

GPCR: Bradykinin

Evidence for the role of bradykinin in myocardial preconditioning comes from the work of Wall et al (100) who evaluated the role of bradykinin in an open chest rabbit model of acute coronary occlusion. Rabbits who received preconditioning (5-minutes ischaemia followed by 10-minutes reperfusion) or an intra-arterial

infusion of bradykinin were protected from the insult of a 30-minute coronary occlusion. This was abolished in the presence of HOE 140, a potent bradykinin receptor antagonist (100).

Other triggers of G protein coupled receptors include angiotensin II (101), catecholamines (102), endothelin (103), urocortin, adrenomedullin and glucagon-like peptide (104).

Growth factor receptors

Growth factor receptors have also been implicated in triggering cardioprotection (105). Examples of ligands acting on this receptor include insulin, insulin-like growth factor, fibroblast growth factor, granulocyte colony stimulating factor, erythropoietin and adipocytokines (95). Atrial natriuretic peptide (ANP) has been shown to trigger ligand specific ANP receptors (106). Furthermore, mechanical stimuli (107) and heat (89) have been described to trigger preconditioning amongst other substances including volatile anaesthesia, statins and metformin (95).

Although numerous substances listed above act to mimic ischaemic preconditioning, blocking their receptors does not necessarily lead to blocking their preconditioning effects unlike those seen in the above

experiments concerning adenosine, opioids and bradykinin. Clearly, ischaemic preconditioning is much more complex than a single trigger being released and occupying its receptor to mediate a protective effect. Undoubtedly, there is a large interplay between these various substances in order to mediate a common effect; cell survival.

It is now known that a preconditioning state can be triggered by any Gi-coupled receptor and as mentioned above, they work in parallel to reduce the preconditioning stimulus. This can be demonstrated by the figure 3 below.

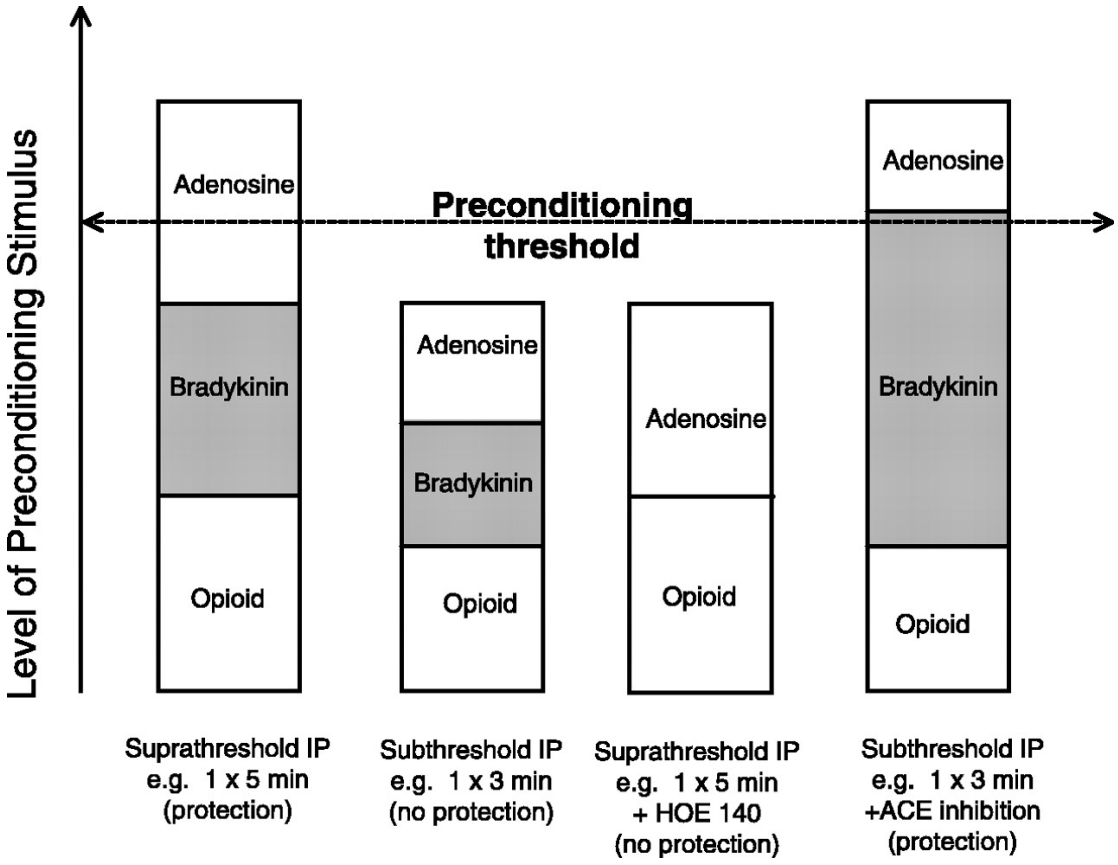


Figure 1.3. Achievement of preconditioning threshold using sub or suprathreshold preconditioning stimuli. Adapted from Morris et al (108).

It can be seen that 5 minutes of ischaemia is required to achieve the threshold for preconditioning (1st panel), but this is not achieved when the duration of ischaemia is lowered to 3 minutes (panel 2). In the 3rd panel, blocking the effects of bradykinin with HOE 140 prevents the suprathreshold 5 minutes ischemia reaching the threshold necessary to protect the cell. Finally, in panel 4, a

subthreshold ischaemic stimulus with 3 minutes hypoxia in the presence of an angiotensin converting enzyme inhibitor (ACEi) that increase bradykinin levels, confers protection. This clearly demonstrates the interplay amongst these receptors. Based upon this, blocking a particular receptor only serves to raise the preconditioning threshold.

Finally, although other Gi receptors are expressed on cardiomyocytes, triggering of which can induce a preconditioning state, these other receptors do not seem to partake in ischaemic preconditioning probably because the agonist to these receptors are not produced in sufficient quantities by the ischaemic myocardium.

1.5.7.2 Intracellular Mediators of Preconditioning

Once preconditioning has been triggered, complex arrays of intracellular cascade of events are enrolled that recruit a series of prosurvival kinases, which form part of an anti-apoptotic mechanism for cellular protection. These kinases undergo some form of post-translational modification and then convey cardioprotective signals to the mitochondria that leads to the generation of reactive oxygen species that activate protein kinases such as Akt, Erk 1/2, protein

kinase C and tyrosine kinase. These provide the required memory allowing the ischaemic preconditioning effect to last the initial few hours as seen in classical preconditioning, which is the focus of this thesis. To achieve the second window of protection, a series of transcription factors (AP-1, hypoxia-inducible factor 1 alpha, nuclear factor kB, nuclear factor erythroid2-related factor 2) and signal transducer and activator of transcription (STAT) become activated by these kinases to enable the production of distal mediators (such as prostaglandin G/H synthase (COX-2), heat shock proteins e.g. Hsp72 and inducible nitric oxide synthase) that produces the second window of protection, 12-24 hours following the initial preconditioning stimulus. It is now known that the protection afforded through ischaemic preconditioning is mediated through several signalling pathways including the reperfusion injury salvage kinase pathway (RISK- comprising of PI3-kinase/Akt and Erk) and the Survivor Activator Factor Enhancement (SAFE) pathway (comprising – TNF alpha, JAK-STAT 3) (109)

In this regard, the activation of phosphatidylinositol-3-OH-kinase (PI3K)-Akt and p42/p44 extra-cellular signal-regulated kinases (Erk 1/2), at the onset of reperfusion has been shown to protect cells against the detrimental effects of lethal reperfusion injury. These

innate pro-survival kinases have been collectively termed together and form part of the Reperfusion Injury Salvage Kinase (RISK) pathway, by Yellon's laboratory (110).

Phoshatidylinositol-3-OH-kinase (PI3K)-Akt signalling cascade

In response to various receptor activation, the (PI3K)-Akt signalling cascade is activated where it phosphorylates a whole host of substrates including those involved in glycogen & protein metabolism (glycogen synthase kinase-3), glucose metabolism (GLUT4 vesicles), apoptotic proteins (BAD, BAX, BIM, p53 and caspases), transcription factors, p70S6K, eNOS and PKC (111). Protection against ischaemia-reperfusion injury is achieved through activation of the serine-threonine kinase, Akt, which itself is triggered by PI3 kinase.

The involvement of PI3 kinase was first suggested by Tong et al (112). In their isolated perfused rat heart model, the preconditioned hearts in the presence of a potent PI3 kinase inhibitor, wortmannin, lost protection as demonstrated by a significant reduction in the recovery of left ventricular developed pressure (LVDP) (102). Yellon's group later confirmed this in a similar model group where

they were able to demonstrate the benefits of preconditioning was lost in the presence of inhibitors of PI3-kinase (113).

p42/p44 extra-cellular signal-regulated kinases (Erk 1/2)

Similar to the (PI3K)-Akt signalling cascade, once triggered in the context of ischaemia-reperfusion, protection against cell death is achieved. This family of serine-threonine kinases forms part of the mitogen-activated protein kinases (MAPKs) and is involved in the regulation of cell proliferation, differentiation and survival in response to occupation of G protein-coupled and tyrosine kinase receptors.

The exact mechanism through which damage limitation is achieved remains largely unknown however part explanation is their ability to phosphorylate and inactivate pro-apoptotic proteins. To demonstrate a few, direct or indirect phosphorylation of pro-apoptotic proteins via the RISK pathway, prevents them from binding to its effector mitochondria (BAD), inhibiting some form of conformational shape change that is essential for mediating its pro-apoptotic influence (BAX), reducing the actual expression of pro-apoptotic protein (BIM) or causing their degradation through

intermediaries (p53), thus mediating cellular protection (110). Akt, on the other hand, has been identified to inhibit mitochondrial transition permeability pore opening. By doing so, mitochondrial cytochrome c (required to activate caspases) release from the mitochondrial intermembranous space into the cytosol is inhibited thereby allowing mitochondrial membrane potential to remain unaltered and allowing cell survival. The PI3-Akt has also been shown to activate protein kinase C (PKC) that has been shown to also inactivate pro-apoptotic factors. This partly demonstrates the complex array of cellular reaction that takes place to minimise harm to cells during ischaemia reperfusion injury.

Protein kinase C

Protein kinase C (PKC) is thought to be a common component for many intracellular signal transduction pathways (114). It was Professor Downey and colleagues who identified PKC as the first cytosolic mediator of conditioning whilst investigating the mechanism underlying preconditioning (97). His group was able to counteract the benefits of preconditioning with the use of several PKC inhibitors. It was Downey's group who first proposed that an

endogenous ligand (such as adenosine A1) binding to its specific G protein-coupled receptor, located on cell surface, activated phospholipase C (PLC); the net result was a breakdown of phosphatidylinositol 4,5 bisphosphonate and phosphatidyl choline into inositol 1,4,5-triphosphate and diacyl glycerol (DAG). DAG would then act as a second messenger to cause phosphorylation, activation and translocation of the enzyme PKC. This in turn would phosphorylate a secondary effector leading to the protective effects of preconditioning (105, 115).

Mitogen-Activated Protein Kinases

There are many MAP kinases described in literature (116) amongst which p38 plays an important role in ischaemic preconditioning. This kinase was investigated by Yellon's group (117), where isolated rat hearts in the Langendorff model were subjected to 35 minutes regional ischaemia followed by 2 hours reperfusion. A pharmacological inhibitor of p38 MAPK was administered at various stages of the experiment including during the preconditioning protocol or just prior to and 15 minutes into index ischaemia. They were able to demonstrate that not only p38 MAPK protects the heart

from lethal ischaemia reperfusion injury but the temporal aspect of p38 was crucial; p38 MAPK becomes activated during index ischaemia and therefore the protection afforded by ischaemic preconditioning becomes abrogated when the inhibitor is given during this phase. They were also able to demonstrate that p38 MAPK given in the non preconditioned heart prior to and during the ischaemia had no effect.

Various isoforms of p38 exist (alpha/beta) and have opposing roles. The alpha is thought to become activated during preconditioning and is thought to mediate preconditioning through increased phosphorylation of heat shock proteins.

Survivor Activating Factor Enhancement (SAFE) pathway

The particular pathway involves activation of the cytokine tumour necrosis factor (TNF-a) and the transcription factor signal transducer and activator of transcription-3 (STAT-3) (118). Interestingly, although TNF-a is thought to play a role in heart failure, it paradoxically abrogates myocardial injury during reperfusion, independently of the RISK pathway described earlier (119).

1.5.7.3 End effectors

The mitochondria are thought to be the key mediator of cardioprotection in the context of ischaemia reperfusion injury. They are where most of the signalling cascade merges. Mitochondria are double membrane bound organelles that act as the 'powerhouse' for cells. Through a series of reactions, they produce adenosine triphosphate (ATP) that acts as a source of energy for cells.

The basic structure of the mitochondria consists of an outer mitochondrial membrane that envelopes the entire rod shaped organelle, the intermembrane space, the inner membrane that folds over many times to form cristae, and the matrix that contains fluid.

Mitochondrial Permeability Transition Pore

The mitochondrial permeability transition pore is a channel located within the inner mitochondrial membrane. When the pore is opened, the inner mitochondrial membrane potential is lost leading to matrix swelling, rupture of the outermost membrane and release of cytochrome C from the intermembrane space into the cytoplasm that leads to processes that culminate in cell death. The MPTP was previously thought to be a complex of voltage-dependent anion

channels located on the outer membrane that interacted with adenine nucleotide translocase of the inner membrane, which was regulated by cyclophilin D from within the matrix. The MPTP is now thought to be formed from F0F1 that consists of a F0 domain, which is integral to the inner mitochondrial membrane and the F1 domain located on the periphery of the membrane where the protons are moving. The F-ATPase utilises a proton gradient to drive ATP synthesis. It allows the passive movement of protons along an electrochemical gradient and uses energy released from transport reactions to release newly produced ATP.

During myocardial ischaemia and reperfusion, the MPTP opens resulting in depolarisation of the inner mitochondrial membrane. As with conditioning, paradoxically, transient MPTP opening serves as a physiological function and plays a role in ROS homeostasis and calcium release; and this has been shown to exert a positive cardioprotective effects during ischaemic preconditioning (120). From experimental studies it is clear that cyclophilin D (located in the mitochondrial matrix) is involved in MPTP opening; it lowers the threshold at which MPTP is opened in the presence of calcium and inorganic phosphates. In that regard, cyclosporine A, an inhibitor of cyclophilin D, therefore an inhibitor of MPTP opening, has been

demonstrated to attenuate infarct size in experimental studies in the context of ischaemia and reperfusion. However, less success was achieved in patients in the setting of acute myocardial infarction or during elective coronary artery bypass grafting surgery.

Role of K^+_{ATP} channels in preconditioning

Following the first description of K^+_{ATP} channels in ventricular cardiomyocytes by Noma in cardiac ventricular myocytes (121), importance has been placed upon their role as mediators of preconditioning; this has been demonstrated in experimental models ranging from isolated cells and hearts to whole animals.

It is widely accepted that these channels play a key role in mediating protection and it was Gross and collaborators who first proposed this (122). Studies have since demonstrated its role in preconditioning by producing or inhibiting cardioprotection using the channel openers or blockers respectively. In subsequent experimental studies, adenosine receptor activation has been shown to be upstream of K^+_{ATP} activation as glibenclamide (potent K^+_{ATP} channel blocker) has been shown to attenuate cardioprotection induced through adenosine A1 receptor activation (123).

There are 2 distinct K^+_{ATP} channels, each with unique pharmacological profiles despite similarities in their structure. There are those that reside on the cell surface (sarcolemmal) and those located on the inner mitochondrial membrane (124). For example, 5-hydroxydecanoate has been shown to inhibit the mitochondrial channels at micromolar range but has failed to do the same to the sarcolemmal channels at any concentrations. Similarly, potent openers of K^+_{ATP} channels such as diazoxide are almost a 1000 times more potent opening mitochondrial K^+_{ATP} channels when compared to sarcolemmal channels (125).

An early hypothesis was that cardioprotection was mediated by the sarcolemmal channels by shortening the duration of action potential. We now know that most of the evidence hinges on the mitochondrial K^+_{ATP} as mediators of cardioprotection in conditioning.

Marban and colleagues (126) first identified the involvement of K^+_{ATP} channels, located within the inner mitochondrial membranes in cardioprotection. Opening of the mitochondrial K^+_{ATP} channels will drive K^+ (and water) into mitochondria along its electrochemical gradient (as H^+ ions are extruded); K^+/H^+ exchanger located on the inner membrane then exchange the entered K^+ with extra-mitochondrial H^+. Theoretically, the K^+ entering cells would naturally

uncouple the mitochondrion therefore resulting in reduced ATP production. However the actual degree of uncoupling resulting from this is minimal therefore ATP production is minimally affected. Several theories exist to explain why opening of these K^+ channels is protective, but in essence, the mitochondria are made more resistant to calcium entry.

These particular channels are modulated by intracellular concentrations of ATP and contribute to action potential shortening and prevention of intracellular calcium overload during ischaemia. Therefore low levels of ATP (as seen during ischaemia) causes opening of these channels allowing efflux of potassium ions that hastens the rate of contractile arrest through reducing the duration of action potential and inhibiting calcium entry into cells. This ultimately leads to the preservation of intracellular ATP therefore are protective to cells. In experimental studies, specific K^+_{ATP} antagonists such as the sulphonylureas (Glibenclamide/Tolbutamide) have been shown to block the effects of preconditioning and similarly, opening these channels with agents such as Nicorandil has shown to precondition the myocardium.

Mitochondrial K^+_{ATP} channels are targeted by a variety of signalling moieties including PKC, PKG and NO. Upon activation ROS is released

which then activates PKCe that positively feeds back to cause further ROS release through activation of mitochondrial K^{+}_{ATP}.

1.5.8 Does preconditioning exist in the human heart?

To date preconditioning has been shown to abrogate infarct size in a wide range of experiments and has been successfully reproduced in many species studied but the question remained as to whether this can be applied to humans. Experiments in humans are important because experimental findings on ischaemic preconditioning may not necessarily be extrapolated to humans because of differences in mechanism between species. Evidence for preconditioning in humans mostly come from indirect evidence because logistics and ethical reason prevent us from meeting the strict experimental conditions where preconditioning utilises infarct size as an end point (127). However, Yellon's group were able to demonstrate the efficacy of IPC in humans undergoing coronary artery bypass grafting (128).

There is compelling evidence for the existence of preconditioning in the human myocardium, mainly derived mainly from *ex vivo* experiments involving human atrial trabeculae (129), ventricular

trabeculae (130) and cultured ventricular cardiomyocytes (131). Evidence also arises from studies of patients undergoing elective procedures that invariably involve brief periods of ischaemia such as percutaneous coronary intervention and coronary artery bypass grafting surgery (132). Further proof is also derived from patients who suffer with angina prior to a myocardial infarction and those that experience warm up angina. Some of these are discussed briefly below.

1.5.8.1 *Ex-vivo* model of preconditioning the human myocardium

Atrial trabecular model

The human atrial trabecular model of ischaemia and reperfusion has been developed and studied extensively in Yellon's laboratory at the Hatter Cardiovascular Institute. Briefly, atrial trabeculae isolated from right atrial appendage harvested during cardiac surgery undergo various preconditioning protocols and it has been possible to demonstrate attenuation in ischaemic injury. Some studies have used rapid atrial pacing as a preconditioning mimetic whilst many have used simulated ischaemia as their preconditioning stimulus.

The end points of these studies are reflected by an increase in functional recovery following an ischaemic insult.

Using this model, it has since been possible to exhibit the roles adenosine (129), opioids (133) and bradykinin (108) in preconditioning the human myocardium. Speechly-Dick went onto evaluating the role of protein kinase C and mitochondrial K^{+}_{ATP} channels in the mechanistic aspect of this phenomenon (134) and further studies using the same model have confirmed this by Carr (135). Others have demonstrated the involvement of the RISK pathway in mediating this protection. Shanmuganathan was able to demonstrate the role of MPTP in preconditioning the human myocardium (136). Most recently, Sivaraman was able to show the roles of various isoforms of PKC in preconditioning as well the ability to precondition the more resistant diabetic myocardium using a stronger preconditioning stimulus. The role of the RISK pathway in post-conditioning has also been demonstrated (137). These experiments are summarised below in table 1:

Experimental model	Findings	Reference
3 mins rapid atrial pacing followed by 12 mins reoxygenation	Ability to precondition and involvement of adenosine in PC	Walker DM et al 1995
3 mins simulated ischaemia followed by 7 mins reoxygenation	Role of protein kinase C and ATP dependent K channel	Speechly-D et al 1995
3 mins (or 1.5 mins subthreshold ischaemia) followed by 7 mins reoxygenation	Involvement of bradykinin in PC and interplay amongst various triggers in PC	Morris et al 1997
3 mins simulated ischaemia followed by 7 mins reoxygenation	Role of ATP dependent K channels in PC	Carr et al 1997
3 mins simulated ischaemia followed by 7 mins reoxygenation in patients ± Nicorandil	Role of ATP dependent K channels in PC. Addition of PC abrogates recovery.	Carr et al 1997
3 mins simulated ischaemia followed by 7 mins reoxygenation	Role of adenosine A1 and A3 receptors in PC	Carr et al 1997
3 mins simulated ischaemia followed by 7 mins reoxygenation	Role of delta opioid receptor stimulation in PC	Bell et al 2000
3 mins simulated ischaemia followed by 7 mins reoxygenation	Role of MPTP in PC	Shanmuganathan et al 2005
4 x 60 sec post conditioning	Recruitment of RISK in post conditioning	Sivaraman et al 2007
4 mins simulated ischaemia followed by 16 mins reperfusion	Role of various isoforms of PKC in preconditioning	Sivaraman et al 2009

4 mins simulated ischaemia followed by 16 mins reperfusion	Diabetics requiring a stronger PC stimulus	Sivaraman et al 2010

Table 1.1. Summary of atrial trabecular models at the Hatter Cardiovascular Institute.

Another model using human atrial trabeculae has been developed by Manuel Galinanes based in Leicester where tissue injury is determined by measuring leakage of CK into the incubation medium from sections of atrial appendage harvested during cardiac surgery (138). This group were also able to precondition the human myocardium.

1.5.8.2 Planned Ischaemia during elective procedures

Preconditioning in the context of cardiac surgery

The first study to demonstrate preconditioning exists in the human myocardium comes from our laboratory where we were able to demonstrate this in a cohort of patients undergoing coronary artery bypass grafting (118). In this study, after establishing cardiopulmonary bypass, patients would undergo 2 cycles of

preconditioning by way of 3 minutes of aortic cross clamping interspersed by 2 mins of reperfusion whilst the heart was paced at 90 beats per minute. The index ischaemia was applied when the heart underwent cross clamp fibrillation during distal aortic-coronary anastomosis for 10 minutes. Needle myocardial biopsies taken from the left ventricular free wall were taken at various time intervals including before the application of preconditioning protocol, following the preconditioning protocol and following the ischaemic insult (cross clamp fibrillation). The investigators were able to demonstrate a reduction of ATP in the specimen taken following the preconditioning protocol but more importantly, similar to earlier experiments of Murry et al, were able to demonstrate myocardial preservation of ATP (40). Many small clinical trials have since been conducted to confirm that preconditioning can be applied to the human myocardium through measuring various biomarkers including troponin and creatine kinase. Gaunts' group went on to perform a systematic review and meta-analysis involving 22 clinical trials to evaluate whether preconditioning had any benefits in terms of clinical outcomes. To no surprise, the meta-analysis concluded that although statistically insignificant ($p=0.17$) there was a reduction in death, inotropic use was reduced; postoperative

ventricular arrhythmias were significantly reduced as were the duration of ITU stay (139).

Preconditioning during percutaneous coronary intervention

Percutaneous coronary intervention (PCI) involves balloon inflation of a coronary lesion in order to deliver a coronary stent which itself is mounted onto a balloon which is then inflated, after which the stent becomes deployed. Further optimisation of the deployed stent is achieved by post dilating usually with a non-compliant balloon. During balloon inflations it is common to see that patients go through reducing angina with serial balloon inflations in parallel with a lesser degree of ST segment deviation.

Thus, it is clear that stenting involves brief periods of ischaemia during balloon inflation. Several studies have taken place to evaluate whether this adaptation is due to preconditioning or recruitment of collaterals. It must be borne in mind that such studies only evaluate the normal pattern of reversible injury as seen in coronary angioplasty.

In an earlier study by Deutsch et al (140), the effect of 2 consecutive 90 seconds balloon inflations of isolated left anterior descending

artery (LAD) stenosis in 12 patients undergoing elective PCI was evaluated. Baseline characteristics before initial inflation were similar in all 12 patients and collateral vessels in 3 patients were visible before PCI took place. Parameters observed included haemodynamic response, metabolic, electrocardiographic and symptomatic changes. Amongst the usual cardiac medications, these patients were also given dipyridamole, which is a potentiator of adenosine. Results from this study demonstrated that anginal pain, ST segment elevation and myocardial lactate production were all significantly reduced during the second inflation when compared to the first. In addition coronary wedge pressure during the serial balloon inflations were not significantly different as well as coronary vein flow, which was reduced during the second inflation; therefore these findings suggested that ischaemia resulting from the primary balloon inflation may precondition against subsequent balloon inflations in the absence of recruitment of collateral vessels. This study may also suggest that the duration of ischaemia required to trigger preconditioning may be as low as 90 seconds, which is usually 2.5 minutes as suggested by Downey et al (141). Studies employing shorter durations of ischaemia failed to demonstrate any protection (118).

Cribier et al led a similar study involving 17 patients but their balloon inflations were different involving 5 sequential inflations of 2 minutes duration or more during PCI to LAD artery (142). In 5 patients, baseline angiography identified the presence of pre-existing collaterals. In this study there was improvement in angina and ST segment deviation during sequential inflations however unlike Deutsch's study (128), there was collateral recruitment occurring in 10 patients when collateral flow was compared between the initial and fourth balloon inflation suggesting that this was responsible for the adaptation in ischaemia. Clearly, there were differences in the inflation times used in these studies and it may well be that the ischaemia times of 90 seconds used by Deutsch in their study may have been too low to recruit detectable collateral vessels. Although experimental studies suggest between a 2.5-15 minutes of ischaemia is required to trigger preconditioning, the depth of ischaemia necessary to precondition the human heart is unknown. Deutsch also used a mediator of adenosine that may have potentiated the effects of preconditioning.

1.5.8.3 Does antecedent angina protect against myocardial infarction?

Aside from cardiac surgery, antecedent angina has been considered to mimic a form of ischaemic preconditioning in the setting of an acute myocardial infarction (AMI) and this has been studied extensively. Angina itself is a common manifestation of reversible myocardial ischaemia and is commonly experienced by many patients before an MI. For angina to induce preconditioning, the duration of ischaemia would need to be prolonged (at least 2.5 minutes) and take place within 1-2 hours of the MI as suggested in experimental studies. Therefore unstable angina would be clinically analogous to preconditioning as oppose to chronic stable angina where ischaemia is usually relatively brief and does not necessarily occur within the timeframe prior to an MI. The precise pathophysiology underlying unstable angina is yet to be determined however the formation and dispersion of platelet rich thrombi is thought to play an important role. Experimental evidence suggests that ischaemia resulting from cyclical variation of coronary blood flow as a result of repeated thrombi formation and resolution can induce preconditioning as effectively as mechanical coronary occlusion as applied to original experimental protocols. As we know

from earlier studies i.e. Murrys original paper, preconditioning serves to delay cell death rather than preventing it and Murry was able to demonstrate that extending the index ischaemia to 3 hours from 40 minutes abrogated the effects of preconditioning. For the same reason, investigators have only considered studies only after the advent of thrombolysis, as reperfusion is a prerequisite for preconditioning to take place.

On that note, Muller et al (143) was one of the first groups to study the effect of antecedent angina on clinical outcomes following an acute myocardial infarction and thrombolysis. 775 patients were recruited from the Thrombolysis and Angioplasty in Myocardial Infarction trial (TAMI) and only patients experiencing angina for more than 7 days were grouped in the chronic angina group. The effects of chronic angina prior to an AMI on immediate and 7 days angiographic appearances together with short term and long-term mortality was determined. They were able to conclude that in patients with antecedent angina, the re-occlusion rate of the infarct related artery was lower (8% v 14%) as well as in-hospital mortality (5% v 7%). No difference was seen in the two years mortality data (89% v 90%). Like with many studies, several limitations exist with this type and particular study; firstly these type of studies were not

designed to investigate preconditioning, secondly patients suffering with angina less than 7 days were grouped amongst those without any preceding angina - it may well have been that if these subset of patients were included in the angina group, the benefits of pre-infarct angina may have been more pronounced as patients suffering with bouts of angina immediately prior to their infarct are most likely to have undergone preconditioning, thirdly collateral blood flow was not assessed – this is important because this may be the reason why patients had better short term outcomes. Hirai et al (144) were able to demonstrate this in a group of patients with pre-infarct angina to have better preserved left ventricular function when compared to those without angina but they also had a better collateral circulation at the point of infarction, suggesting that the improved outcomes in the angina cohort was a reflection of better collateral circulation.

Another important factor is that patients in the non-angina arm had a lower incidence of multi-vessel disease and lower risk factor profile. It cannot be deducted solely from this study that angina preconditions the myocardium as during an acute coronary occlusion, it may well be that collateral vessels are recruited and also patients suffering with angina may well have been on agents that improve outcomes following a myocardial infarction. Nonetheless, a

history of angina may well have been of benefit because the 2 year outcome in the angina group was comparable to that of the non anginal group despite the presence of multi-vessel disease and higher risk factor profile (143).

Subsequent larger clinical trial have in fact demonstrated an adverse event in patients suffering with angina prior to their infarct. For example, in an analysis involving more than eight thousand patients in the International rt-Plasminogen Activator/Streptokinase Mortality Trial, Barbash et al (145) discovered the incidence of cardiogenic shock, early reinfarction and in-hospital mortality were higher in patients who suffered with angina prior to their MI. Similar to the trial by Muller et al, patients with angina had significantly higher risk factors than those without. Furthermore, Ruocco et al (146) assessed the effect of antecedent angina in over three thousand patients enrolled in the Thrombolysis in Myocardial Infarction trial II (TIMI II) on the early and late outcomes following an MI. Similar to Barbash's group, reinfarction rate was higher in those experiencing pre-infarct angina compared to those without antecedent angina. There was no difference in the mortality at 21 days amongst the various groups. Authors have concluded that the latter two studies above did not confirm any benefit from pre-infarct angina but rather

exhibit such patients have a worse prognosis likely due to higher incidence of multi-vessel disease and more unfavourable cardiovascular risk factors.

A crucial aspect of preconditioning is the temporal aspect of this phenomenon. None of the aforementioned trials took this into account; Muller included patients into his angina arm as those with more than 7 days, Barbash's angina group divided his angina group into those suffering with angina into those more or less than a month and Ruocco divided his angina cohort into those experiencing angina less than 7 days, 8-180 days and more than 180 days.

To address this, the following clinical trials of pre-infarct angina were designed to determine whether or not preinfarct angina is the clinical equivalence of preconditioning. In the TIMI IV trial by Kloner (147), 350 patients who experienced angina within 48 hours of their infarction, several parameters had improved. Infarct size, as determined by total CK release, was significantly less, as were cardiogenic shock, severe heart failure and in-hospital mortality. Importantly, in this study, angiography was performed 90 minutes after initiating thrombolysis to determine collateral burden in the infarct related artery; the authors concluded no difference between both groups. A further smaller study conducted by Ottani et al (148)

involving only 25 patients found no difference in collateral circulation at three weeks following intravenous thrombolysis and there was also a reduction in infarct size (lower CK-MB release), coupled with a reduced degree of hypokinesia on ventriculography in patients experiencing angina within 24 hours of their MI.

Despite addressing the temporal aspects of antecedent angina, an important limitation yet existed and this was the issue relating to collateralisation. An important determinant of infarct size in relation to coronary artery occlusion is the presence of collateral vessel and also patients who experience angina more than a week prior to their infarct have a high incidence of collateral blood supply to the infarct related arterial territory. Therefore the estimation of collateralisation in the studies by Kloner and Ottani may actually be underestimated as angiography was performed in the absence of complete coronary occlusion i.e. following intravenous thrombolysis. Therefore the presence of significant collateral supply during the peri-infarct period remains unknown. It is unlikely that collateral vessels would have formed in the latter 2 studies but it is unclear from these patients whether they had a history of chronic ischaemia as this would lead to the formation of collaterals. Finally these studies relied on patients self reporting angina - this reiterates the

fact that these studies are not designed to test preconditioning as the self reported symptoms of angina does not really match the intermittent ischaemia-reperfusion protocols employed during original experimental models.

Warm up angina and preconditioning leading to walk through angina

Akin to preinfarct angina, preconditioning may also occur during this paradoxical phenomenon of walk through angina. In essence, patients report the ability to exert themselves until they experience anginal symptoms (first wind/warm up/first hole angina) and following a brief rest period, they are able to continue to exert themselves without any further or minimal limitations (walk through/second wind angina). William Heberden first described this more than two centuries ago in 1772 following which it was first reviewed in the British Heart Journal in 1951 and then again by our laboratory in 1994 by Marber et al (149). Interestingly 1 in 5 patients report walk through angina and objective testing has shown patients to have enhanced performance during second effort. The paradox that that patients are resistant to further exertional angina

following a brief rest period following initial period of effort bears a resemblance to the paradox of preconditioning. Despite its longstanding recognition, like many things in science, the mechanism underlying this phenomenon remains indeterminate. However several mechanistic principles have been proposed including:

(i) Increased myocardial resistance to ischaemia akin to ischaemic preconditioning,

(ii) An improvement in myocardial perfusion and

(iii) A reduction in myocardial work.

Other forms of conditioning

1.6 Postconditioning

A number of the major challenges faced with preconditioning are its pre-requisite to apply this prior to an index event i.e. an MI. In reality this is unrealistic as an MI is unpredictable and preconditioning is a usually a short-lived phenomenon. This remained a scientific mystery until the discovery of post-conditioning where repetitive occlusion and reperfusion within minutes during early

revascularisation provided the same powerful protection as preconditioning shown in a landmark study by Zhao and co-authors in 2003 (150). In their landmark study they used an open chest canine model, where dogs were subjected to:

- Control protocol of 60 minutes LAD ligation followed by 3 hours reperfusion
- Preconditioning protocol where the LAD was occluded for 5 minutes then reperfused for 10 minutes prior to the index ischaemia for 60 minutes
- Post conditioning protocol of three cycles 30 seconds of reperfusion and occlusion following the index ischaemic insult

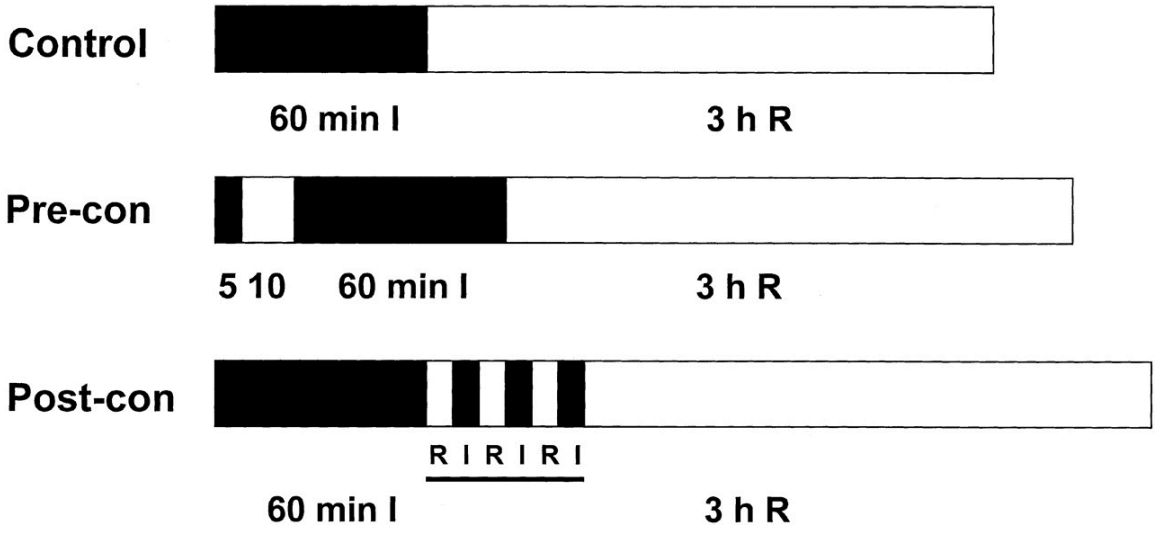


Figure 1.4. Protocol used by Zhao et al during preconditioning (143).

Infarct size was considerably lower in both the preconditioned (14%) and postconditioned (15%) hearts when compared to control (25%) hearts. Furthermore polymorphonuclear accumulation in the area at risk myocardium was lower in comparison to the control group. Other improved parameters included improved endothelial function (post ischaemic response of LAD to acetylcholine). Based on these parameters, authors were able to conclude that post conditioning is as effective as preconditioning in reducing infarct size and preserving endothelial function and therefore can be applied to circumstances where ischaemia was involved such as coronary intervention, coronary artery bypass surgery, organ transplantation

and peripheral revascularisation. The same protection was also identified to be present by the same group in rats (151).

It was not long before Staat and co-authors in 2005 were able to apply the principles of post conditioning from bench to bedside in their pioneering proof of concept study applied to humans (152). In this study, patients presenting with an AMI were subjected to either to a control (coronary occlusion treated by direct stenting) arm or an intervention arm where within one minute of establishing flow down the infarct related artery, they were subjected to further intervention by means of 4 cycles of one minute inflation and one minute deflation using the angioplasty balloon. Baseline characteristics were comparable in both groups and creatine kinase release was measured over 72 hours and presented as the area under curve of creatine kinase release AUC-CK. Analysis confirmed a reduction in the AUC-CK in the postconditioned group when compared to control subjects (equating to a relative risk reduction of 36%) thus confirming the concept that postconditioning by coronary angioplasty protects the human heart during an AMI. Since then a number of clinical trials have taken place to confirm that postconditioning exists. Besides reducing infarct size, postconditioning has also been shown to reduce other detrimental

effects of reperfusion injury including endothelial dysfunction, post-ischaemic blood flow defects (no reflow phenomenon) and contractile dysfunction.

From a mechanistic perspective, both pre and postconditioning share similar pathways and is referred by Heusch as “old wine in a new bottle” (153). It is thought that the intervention applied at the time of reperfusion must act against the known mediators of lethal reperfusion injury, namely calcium paradox (cellular and mitochondrial calcium overload), burst of oxidative stress, endothelial dysfunction and reduced nitric oxide production. In our laboratory we were able to demonstrate that the same fundamental pathways are activated, that include RISK pathway comprising phosphatidylinositol 3-kinase-Akt and extracellular signal-related kinase. It has been suggested that there is upstream activation of GPCR and downstream phosphorylation of eNOS and inhibition of apoptosis promoters. As with preconditioning, these fundamental pathways converge onto the mitochondria, in particular the MPTP as the end effector, to inhibit pore opening that is initially promoted during the early stage of reperfusion, in response to mitochondrial calcium overload, oxidative stress and ATP depletion.

Following the initial proof of concept study by Staat and co-workers, several randomised trials of postconditioning in ST-elevation myocardial infarction have taken place and most recently in a meta-analysis involving fourteen randomised controlled trials totalling 778 patients, Favaretto and colleagues (154) were able to conclude postconditioning to be cardioprotective in terms of infarct size reduction, however significant differences existed between these trials but univariate meta-regression analysis did not identify clinical or procedural variables to be associated with a more pronounced effect of postconditioning effects on the infarct size with the exception of cardiac magnetic resonance. Further analysis of 6 RCTs that used CMR to evaluate the effects of postconditioning on infarct size led to the conclusion that postconditioning had a limited benefit on infarct size suggesting that further data was necessary before postconditioning could be rolled out into clinical practice.

So far, I have discussed benefits of preconditioning in experimental models of infarction or have used pre infarct angina as a surrogate marker of ischaemia preconditioning in those suffering from an MI. Similarly discussions have taken place regarding postconditioning. Both advantages and disadvantages have been eluded upon however

a common theme to both these interventions is the invasive stimuli requirement needed to trigger protection e.g. either ligation of infarct related artery prior to infarction (preconditioning) or repeated inflation/deflation of an angioplasty balloon (post conditioning). Although concerns have been raised in the latter (injury to infarct related artery through repeated balloon inflations/dissection/migration of atheromatous material), application of postconditioning has been deemed safe with no post stenting complications reported. The method of application has therefore limited its use clinically. An innovative strategy to confer cardioprotection from brief episodes of ischaemia and reperfusion applied to a distant organ or tissue prior to a period of sustained ischaemia provided an alternative strategy for mediating protection- this has been termed Remote Ischemic Preconditioning (RIC). Although my research is not directly related to RIC, this is mentioned here for completeness.

1.7 Remote Ischaemic preconditioning

Definition

Remote ischaemic preconditioning (RIC) describes an intriguing phenomenon where reversible episode of sub-lethal ischaemia and reperfusion applied in one vascular bed, tissue or organ confers global protection, rendering remote tissues and organs resistant to the detrimental aftermath of lethal reperfusion injury (79).

Initial discovery

It was Przyklenk and co-authors that first described this fascinating phenomenon in 1993 in their seminal paper "*regional ischaemic preconditioning protects remote virgin myocardium from subsequent sustained coronary occlusion*" (79). In their groundbreaking experiment, anaesthetised dogs underwent four episodes of 5-minutes circumflex coronary artery occlusion followed by 5-minutes reperfusion, followed by 1 hour of sustained LAD coronary artery occlusion before 4.5 hours reperfusion. Infarct sizes of the circumflex preconditioned group of dogs were significantly less than

that seen in the control group introducing the concept that virgin myocardium could be protected from subsequent coronary occlusion by brief cycles of sublethal ischaemia-reperfusion injury (79). It was also noted by the investigators that segmental shortening in the myocardium subtended by the LAD in the circumflex preconditioned dogs were preserved compared to control despite having comparable subendocardial blood flow (79). In addition to the initial conclusion, authors also suggested that RIC is mediated by factors that are produced, activated and transferred within the heart following ischaemic preconditioning to protect a virgin myocardium from subsequent sustained ischaemia (79).

Widening the concept of RIC

Following the initial discovery of RIC, multiple in vitro and in vivo experimental models have since demonstrated the existence of this. Using an isolated perfused heart model, Dickson et al (155) collected and transferred coronary effluent from 2 groups of 'donor' rabbit hearts (one group underwent a standard preconditioning protocol) and perfused 2 groups of 'acceptor' rabbit hearts that subsequently underwent 40 minutes of global ischaemia. The authors noted that

the preconditioned donor hearts and acceptor hearts receiving the coronary effluent from the preconditioned donor hearts were all protected from the detrimental effects of global ischaemia with no difference in the magnitude of infarct size observed between the preconditioned hearts and the acceptor hearts receiving the effluent (155). Following these earlier observations, further evolution of the model has taken place and similar results have been observed when the serum from preconditioned animals have been applied to isolated hearts or cultured cells (156-158) thus providing evidence for the existence of cross-species protection RIC including treatment of isolated buffer-perfused hearts of rabbits using human serum (156, 158). From models of intra-organ or intra-myocardium RIC, further experimental studies have now led to the discovery that cardioprotection can also be achieved when the remote stimulus is applied to a tissue bed or organ remote from the heart; Preliminary evidence stems from the laboratory curiosity by Gho et al (159) who hypothesised that ischaemia in remote organs may protect against an MI. They were subsequently able to demonstrate the attenuation of MI size when the mesenteric artery or renal artery was briefly occluded prior to inducing an MI by 60 minutes of coronary artery occlusion (159). They were also able to conclude that RIC recruits a

neurogenic pathway, as hexamethomuin (a potent ganglion blocker) blocks the protection afforded by inter-organ RIC but not with intra-organ RIC (159). Further experimental work have since taken place to provide evidence for the existence of RIC induced myocardial protection when the RIC stimulus is applied remotely from the heart including skeletal muscle, intestines and kidneys – a concept now known as inter-organ RIC. We now know that RIC exists in organs other than the heart to include the liver, kidney, intestine, brain and other tissues; this is summarised in our recent review (160).

While these discoveries are innovative, their translation from bench to practice has been limited by its invasive nature; the intriguing break through came from the discovery that transient limb ischaemia could also exert similar cardioprotection to that seen with remote ischaemic preconditioning. A group led by Kloner in 1997 (161) examined the hypothesis that the heart can be preconditioned at a distance by partial reduction of blood flow to the hind limb with or without increase of demand by electrical stimulation of a skeletal muscle. In their seminal discovery, anaesthetised rabbits were randomised to a time-matched control (30 minutes waiting period), 55-65% reduction of femoral arterial blood flow, electrical stimulation of the gastrocnemius muscle at 1 Hz or a combination of

the latter two. They were then subjected to 30-minutes of coronary artery occlusion followed by 4 hours reperfusion. They found that the ratio of infarct size to risk zone was smaller in the group where gastrocnemius stimulation was combined with a reduction of femoral artery blood flow (161). Oxman and colleagues (162) pioneered this concept further and subsequently were the first group to demonstrate a reduction of reperfusion related arrhythmia in an isolated rat heart model through application of a non-invasive remote preconditioning stimulus (10 minutes hind limb ischaemia using a simple tourniquet). While many studies use infarct size as an end point, this particular study examined the effect on arrhythmias, (as one of the cardioprotective properties of preconditioning is the anti-arrhythmic property of this prodigy). A further notable example is perhaps a porcine model of inter-organ RIC where brief periods of hind-limb ischaemia using a simple tourniquet reduced MI size; this opened doors to subsequent phase II trials in humans to test the efficacy of cardioprotection by applying RIC to a limb. In an important study by Kharbanda et al, 15-kg pigs were subjected to 40 minutes LAD balloon occlusion preceded by four 5-minutes cycle of lower limb ischaemia that resulted in an astonishing reduction of infarct size from 53% to 26% (163). In a parallel study conducted in

humans conducted by the same group, preconditioning the upper limb by three 5-minutes of ischaemia interspersed with reperfusion using a blood pressure cuff inflated to 200 mm Hg reduced the endothelial injury induced by sustained 20 minutes of ischaemia in the contralateral limb – this was demonstrated using venous plethysmography to demonstrate forearm blood flow in response to acetylcholine.

While many of these experiments were confirmatory studies for the existence of RIC, they too also explored the mechanism recruited to allow such phenomenon to exist; furthermore the possibility of being able to apply such stimulus remotely and non-invasively has paved way to explore RIC in humans in various clinical settings.

1.7.1 Application of RIC in the clinical setting: CABG

With the ability to apply RIC non–invasively, application during elective CABG/PCI procedures or during an acute myocardial infarction has become promising. At present, with the ageing population, cardiac surgery is increasingly being carried out in this age group who are naturally at higher risk amongst the general population who now have increased comorbidities. Previously such

patients would be declined surgery, however, with combined progression of anaesthetics, perioperative techniques and surgical procedures, operating on such patients has now become feasible. Despite these advances, there still remain adverse outcomes related to periprocedural myocardial injury (such as reduced left ventricular ejection fraction) therefore further protective measures are necessary. In this regard, RIC has been applied to such settings. Many of these studies have developed the concepts introduced by Kloner (hind limb ischaemia) and MacAllister (forearm ischaemia) to apply this to clinical studies and use biomarkers as an endpoint in cardiac surgery. Many of these studies have shown cardioprotective potential with similar findings in patients undergoing elective PCI. Although widely explored, discrepant results have been noted amongst these studies using RIC and as a result some of these studies have been reported as being negative (164). This can partly be explained by the presence of type II errors including concomitant therapy with beta-blockers or statins which have been shown to be cardioprotective as well as anaesthetic regimes using propofol or volatile anaesthesia that can potentially interfere with RIC (164).

In recent larger clinical studies, besides surrogate markers of cardioprotection, investigators have looked into long-term clinical

outcomes and have deduced a reduction in major adverse cardiovascular outcomes in both elective CABG surgery (165) and PCI (166). More recently, in the first outcome study of its kind an improvement in patients undergoing elective on-pump CABG with or without valve surgery was not observed. This may be partly due to being underpowered, missing data and the presence of propofol (cardioprotective agent)(167).

1.7.2 Application of RIC in the clinical setting: AMI

Similar to elective procedures noted above, advances in treatment of MIs have led to a substantial improvement in 30-day morbidity and mortality despite ischaemic heart disease maintaining its position as the global leading cause of death. Although RIC has been applied prophylactically in experimental models, this is not a requisite for cardioprotection; reduction in infarct size have been reported when the remote stimulus has applied during the index event (remote ischaemic perconditioning) and at the time of reperfusion (remote ischaemic postconditioning). Konstantiov (168) provided the earlier practical evidence of the infarct sparing effects in a swine model thus providing the rationale for the landmark clinical trial of acute MIs

where limb ischaemia was applied to the infarcting patients on route to hospital (169). Clinical evaluation of infarct size can be estimated indirectly using biomarkers released from tissue and from the resolution of ST-segment on surface ECG. A more direct approach entails using myocardial perfusion imaging or cardiac magnetic resonance imaging from which the salvage index (proportion of salvaged area at risk) can be calculated. Using this principle, the important study led by Botker (169) were able to demonstrate RIC applied before hospital admission increased myocardial salvage by 36%, thereby reducing final infarct size in patients during an evolving MI. Furthermore, those with LAD occlusion as seen in anterior MIs and those with complete occlusion of the infarct related artery had an infarct size reduced to 44% and 31% respectively compared to PCI alone. This translated to an improvement of left ventricular systolic function and when followed up, these patients were found to have a reduction in MACE at 4-years after the index event. A similar study by Rentoukas et al has also shown full ST-segment resolution coupled with a significant reduction in troponin release when RIC employed during a PPCI (170). Of the aforementioned studies, whilst precedence has been given to the application of non-invasive application of RIC in various clinical

settings, a vast number of these studies have also suggested a potential mediator/mechanism for RIC.

1.7.3 Potential mechanisms for RIC

1.7.3.1 Triggers

In preliminary studies, RIC has been initiated by a brief ischaemic stimulus. However, evidence from multiple in vivo and in vitro models have suggested that transient ischaemia is not obligatory to trigger remote cardioprotection (164). These include peripheral nociception (sensory nervous systems response to unpleasant stimuli) such as those initiated by skin incision (termed remote pre-conditioning of trauma) (171-173), direct peripheral nerve stimulation, non invasive transcutaneous nerve stimulation (174) and electroacupuncture (175).

1.7.3.2 Signal Transduction

Signal transduction from the remote tissue or organ is thought to involve the nervous system (consisting of somatosensory system, spinal cord and autonomic nervous system) that is initiated by not only by local ischaemia/reperfusion injury in an organ other than the heart (limb, mesentery), but also due local nerve injury or stimulation by capsaicin (171, 176, 177), adenosine (178), and bradykinin (173, 179) to produce cardioprotection whilst RIC has been abrogated in the presence of sensory nerve blockers (176) or local anaesthetic (171). Interestingly, femoral nerve transection did not eliminate limb RIC in a mice model (180). The nociceptive stimulus is thought to involve protein kinase Cy (173) and is inhibited by donors of nitric oxide in rat models of RIC (178).

It has become evident that RIC involves activation of peripheral nerves but the way in which this signal is transferred to heart remains equivocal (164). There is a suggestion that a neuronally released signalling molecule is transferred to the heart humorally (164). This is evident from models where coronary effluent from a donor-preconditioned heart has protected naïve hearts from ischaemia (155) and similarly from porcine transplant models where the preconditioned recipient receiving the denervated donor heart

was protected from an MI (168). Subsequent studies have shown this to be a hydrophobic low molecular mass (<15-kDa) circulating molecule (156). There have been suggestions that this molecule may be nitric oxide but studies have revealed mixed results. More recently, the small chemokine, stromal derived factor-1 alpha (SDF-1α) through its interaction with chemokine receptor 4 (CXCR-4) has emerged as a possible putative factor in conditioning. A recent study by Davidson et al has shown increased circulating levels of this chemokine in rats subjected to RIC by limb ischaemic-reperfusion where reduced infarct size was seen. This was blocked (at least partially) in the presence of the chemokine receptor 4 (CXCR4) inhibitor, suggesting SDF-1α has a role to play in RIC amongst other factors (181).

1.8 General introduction: Stromal Cell-Derived Factor-1α

Stromal cell-derived factor-1α (SDF-1α) has recently become the focus of intense interest and discussion with regards to ischaemia-reperfusion injury with growing body of evidence to support this. It is thought to acutely protect the myocardium from the deleterious effects of ischaemia reperfusion injury through activating cell survival signalling pathways whilst simultaneously recruiting stem cells to the injured site to reduce myocardial injury and dysfunction (182). Additionally SDF-1α has been shown to play a pivotal role in stem cell proliferation, retention, survival, cardiomyocytes repair, neoangiogenesis and ventricular remodelling following myocardial infarction (183-186). This occurs through interaction with its cognate receptor CXCR4, which is a G-protein coupled receptor; this SDF-1α – CXCR4 axis is upregulated in experimental and clinical models of MI leading to reduction of ischaemia burden and preservation of ventricular function. Interestingly SDF-1α – CXCR4 axis exert these effects via the same Gαi dependent mechanisms responsible for conferring protection against ischaemia-reperfusion injury seen in other form of conditioning including pre, post and remote ischaemic conditioning and involves activation of phosphoinositide 3 kinase (PI3K), mitogen activated protein kinase

(MAPK), and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signalling.

It is therefore proposed in my thesis, that in addition to the pleiotropic effects of SDF-1α mentioned above, it has a direct role in protecting the human myocardium. Evidence for this is discussed below.

1.8.1 Chemokines and their structure

Chemokines or chemoattractant cytokines such as SDF-1α are a large group of secreted proteins with a molecular weight between 8-14 kDa that share a structural homology in up to 90% of cases (187, 188). There may be as many as 40-50 human cytokines (189) and their classification into 4 subfamilies is based upon the relative position of the two N-terminal cysteine (amino acid) residues that are bonded to two other cysteine residues via disulphide bonding (190).

All chemokines are similar in that they are composed of at least 3 beta pleated sheets (β1-3) and a C-terminal α-helix (figure 5).

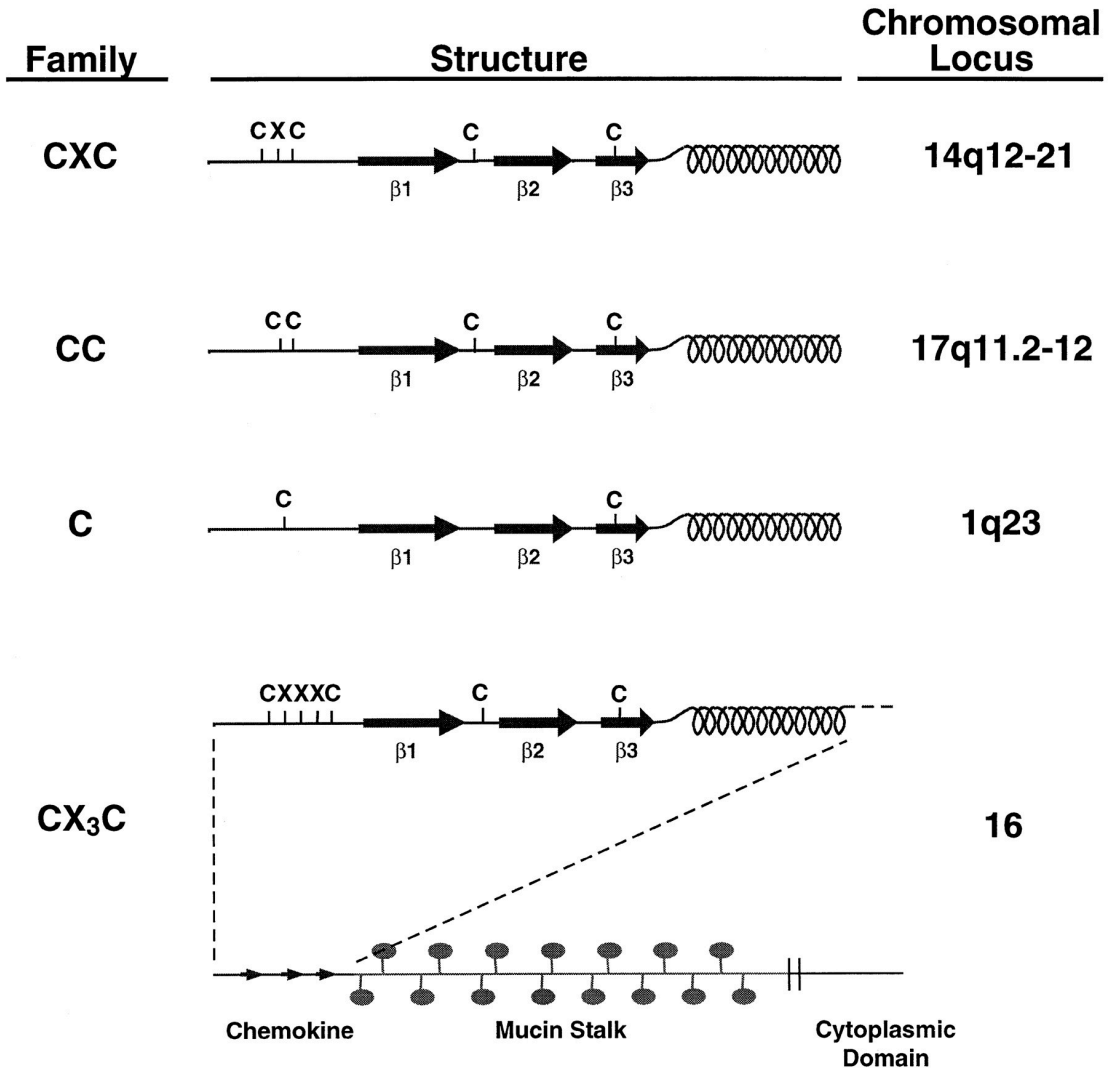


Figure 1.5. Basic chemokine structure composed of 3 β pleated sheets and a C-terminal α-helix.

Chemokines in general contain four cysteine residues in highly conserved positions (191). SDF-1α (also recognised as CXCL12) consists of 89 amino acids and belongs to the CXC chemokine family where the two N-terminal cysteine residues are separated by a single amino acid. Unlike other chemokines of the CXC sub-family which

are clustered, SDF-1, coded by the CXL gene, is uniquely localised on chromosome 10 (192) and is expressed in many cell types including the heart, bone marrow, liver, kidney, thymus, skeletal muscle and brain (193-195). The gene encoding SDF-1α was first cloned from mouse bone marrow stromal cells, hence the name 'stromal cell derived factor – 1 α' (33, 196). Furthermore, the CXC chemokines may be subdivided into either ELR^+ or ELR^- based upon the presence of the sequence of a particular amino acid (glutamic acid-leucine-arginine); SDF1/CXCL12 is an example of the former and is capable of recruiting monocytes and lymphocytes (186). In addition to cell homing, chemokines contribute an important role in apoptosis, gene transcription, mitogenesis and degranulation (189). They have also been implicated in multiple disease processes including cancers (197, 198), infection with HIV (199) and multiple inflammatory states (200, 201). Deficiency of SDF-1α or CXCR4 receptor in mice experimental models has been shown to cause embryonic lethality due to serious structural defects including anomalies in gastrointestinal blood vessel formation and ventricular septal defects (193, 202, 203). Additionally, production of blood cells within the bone marrow (haematopoiesis) have been shown to become compromised with decreased bone marrow colonisation of

haemopoeitic stem cells, development deficiencies of B cells and decreased myelopoeisis (185).

Various isoforms of SDF-1 exist in humans (and mice) including SDF-1α (89 amino acids/3 exons) and SDF-1β (93 amino acids/4 exons) that are encoded by the same gene on chromosome 10 but are produced by alternative splicing (204). Other spliced variants of SDF-1 exist in humans including SDF-1ζ, SDF-1δ, SDF-1ε (205, 206), however the functional diversity of these SDF-1 variants are yet to be fully understood.

SDF-1α is cleaved by several exopeptidases, such as, dipeptidyl peptidase-4 (DPP4), matrix metalloproteinase (MMP)-2, and 9 (206). Proteolysis of SDF-1 by DPP4 takes place at position 2-3 whereas that by MMP occurs at position 4-5; this leads to the release of the N-terminal tetrapeptide, culminating in the loss of binding of SDF-1 at its binding site on the CXCR4 receptor and inactivation of the molecule (207). Although several exopeptidases exist, DPP4 is thought to prevail in this manner (208). Interestingly, when compared to SDF-1α, the other isoforms of SDF-1 are more resistant to the proteolytic degradation (209).

Besides having structural homology amongst chemokines, SDF-1α is highly conserved between species and therefore in experimental models, protection can be transferred amongst them (192).

1.8.2 SDF-1α and receptor interaction

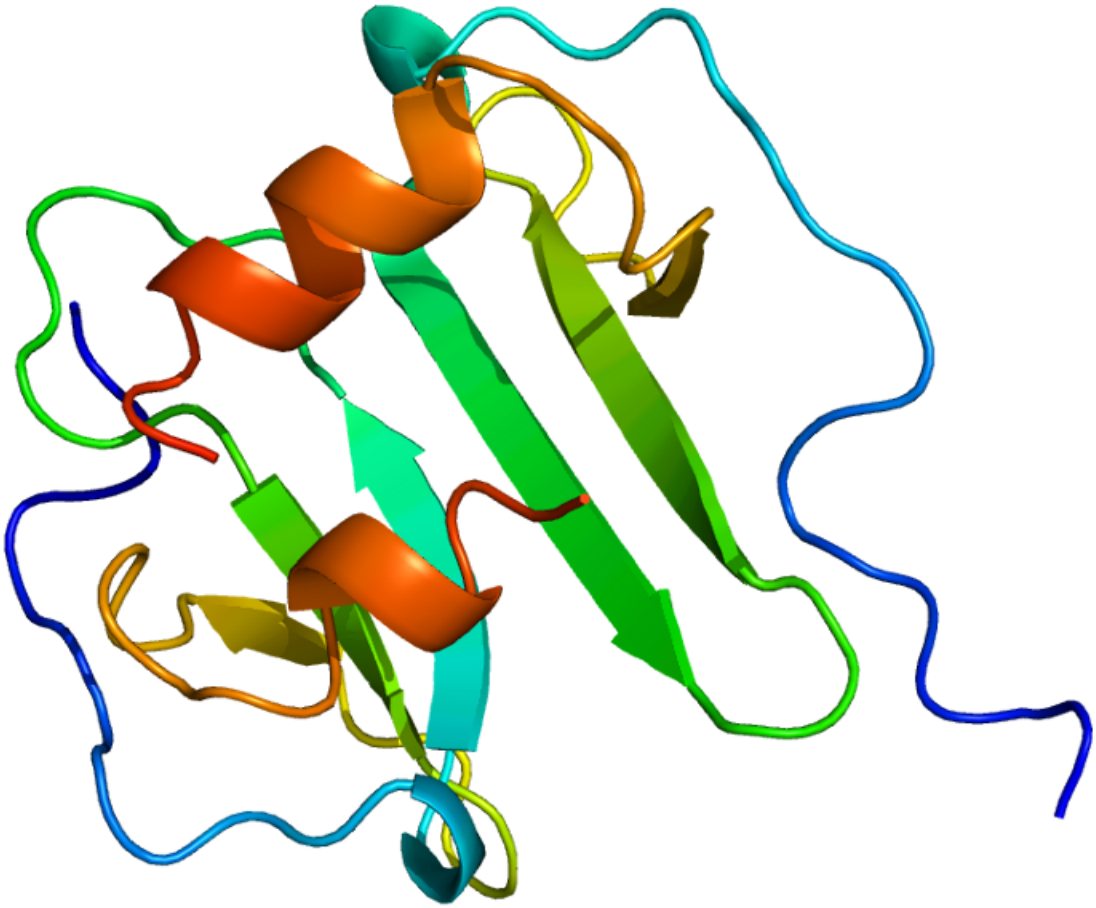


Figure 1.6. Structure of SDF-1α

SDF-1α exerts its action through interaction with specific CXCR4 chemokine receptors found on cell surfaces of specific cell types including cardiomyocytes, vascular and haematopoietic progenitor

cells (185). These are G-protein coupled receptors. SDF-1α binds to its receptor site via its N-terminal which are composed of eight amino acid residues then docks via amino acid residues 12-17 located in the loop (acts as the docking binding site) (210). Once bound, it exerts its biological effects, which are described below.

CXCR4 receptor interaction

The interaction between SDF-1 and CXCR4 receptor on target calls leads to the activation of multiple downstream signalling pathways resulting in a wide array of biological effects including chemotactic response (211, 212). This is summarised in the table 2 below.

Biological effects of SDF-1α	
• Chemotaxis & adhesion • Motility • Secretion of metalloproteinase (MMP) and angiogenic factors	• Retention and recruitment of bone marrow stem/progenitor cells • Angiogenesis

Table 1.2. Biological effects of SDF-1α.

The binding of SDF-1 to CXCR4 results in G-protein coupled receptor activation through homo- or hetero- dimerization (213). This leads to G*a*i protein pathway activation culminating in the regulation of several signalling cascades including and not limited to the activation

of PI3K-AKT, MAPK and Jak-STAT (211, 212, 214, 215). This is depicted in the diagram below (figure 1.7).

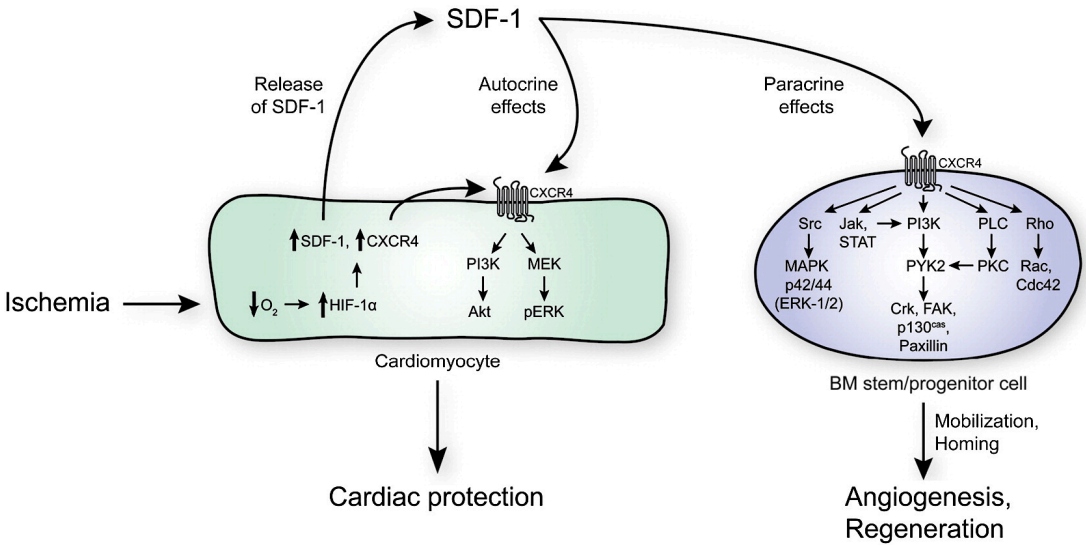


Figure 1.7

Binding of SDF-1 to GPCR CXR4 induces a variety signalling pathways in different cell types; in cardiomyocytes, the activation of PI3K and AKT pathways lead to enhanced cell survival whilst in bone marrow stem/progenitor cells, activation of the various pathways result in the activation and release of BM stem/progenitor cells into the peripheral blood and homing to sites of tissue injury where they participate in tissue repair and neoangiogenesis (186).

It has now been proposed that activation of the second SDF-1 receptor may also regulate MAPK, PI3K, Jak-STAT cascades leading to the homing of progenitor cells at injured sites (216).

CXCR7 receptor

The initial belief was that SDF-1 was an exclusive ligand for the G-protein coupled receptor, CXCR4, highlighting its unique biological role. However, further studies have demonstrated the interaction of this chemokine with another receptor, CXCR7/RDC1 (217), making the biological role of SDF-1 more complex and difficult to understand in stem cell homing as seen when SDF-1 interacts with CXCR4 (218, 219).

It has now been shown that CXCR7 receptors are present in a wide variety of cell types including activated endothelial cells, foetal liver cells, T cells, B cells and renal multi-potent progenitors (217, 220). However the function of this receptor remains unclear and controversial in that some authors suggest that it is a non-functioning decoy receptor and is unable to activate intracellular signalling cascades whereas others suggest that it is a true signalling receptor with the capacity to activate certain pathways involving mitogen-

activated protein kinases (p41/42) and phosphorylation of AKT through receptor binding (221, 222). Interestingly, it may play a regulatory role for SDF-1α; briefly, under hypoxic conditions, CXCR7 becomes up regulated by HIF-1α similar to that described in the paradigm above and is thought to scavenge SDF-1α (223). Hoffmans group was able to demonstrate the rapid internalisation of SDF-1α, degradation and release of by products (223). Conversely, SDF-1α levels have been elevated in murine models where there was pharmacological inhibition or genetic deletion of the CXCR7 receptor. Besides hypoxia, CXCR7 receptor has been reported to be up regulated in various disease states including cancers, autoimmune and inflammatory conditions (224). The interplay between CXCR4 and CXCR7 receptors may be complex and their contribution to cardioprotection is yet to be clarified. It must therefore be borne in mind that any actions of SDF-1α described in this thesis assumes that via CXCR4 interaction which of course may be a confounding factor when considering the actions of SDF-1α.

1.8.3 Role of Stromal Cell-Derived Factor-1α in MI

We now know that several chemokines become upregulated in experimental models of myocardial infarction (225). These include both CXC (interleukin-8 (226-229), growth related oncogene alpha (227) + IP-10) and CC (CCL2 (230, 231) and macrophage inflammatory protein-1 α/β (232)) subtypes of the chemokine family. In particular experimental models where SDF-1 was shown to be upregulated include studies by Ma J (233), Abbott JD (234) and Pillarisetti K (235). To demonstrate these two experimental models of MI are employed, namely the ischaemia-reperfusion injury model and the permanent myocardial infarction model (236, 237). Key differences are summarised in table 3 below:

Ischaemia-reperfusion model	Permanent MI model
Large release of reactive-oxygen species resulting in reperfusion injury	Smaller release of reactive-oxygen species
Larger inflammatory response	Smaller inflammatory response
Accelerated collagen deposition resulting in improved tissue healing (determined by inflammatory response)	Collagen deposition to a smaller degree resulting in a relatively lesser tissue healing
Enhanced neovascularisation	

Table 1.3. Differences between IR and permanent MI models.

Furthermore, in keeping with experimental models of MI, chemokines including SDF-1 have been shown to be elevated in serum in patients with MI (238). This highlights the significance of chemokines in the pathophysiology of myocardial infarction.

Upregulation of chemokines in the infarcted heart

Several transcription factors are know to be implicated in the upregulation of chemokines; one prime example includes hypoxia-inducible factor-1α (HIF-1α) (239, 240) as depicted in the figure 1.8 below:

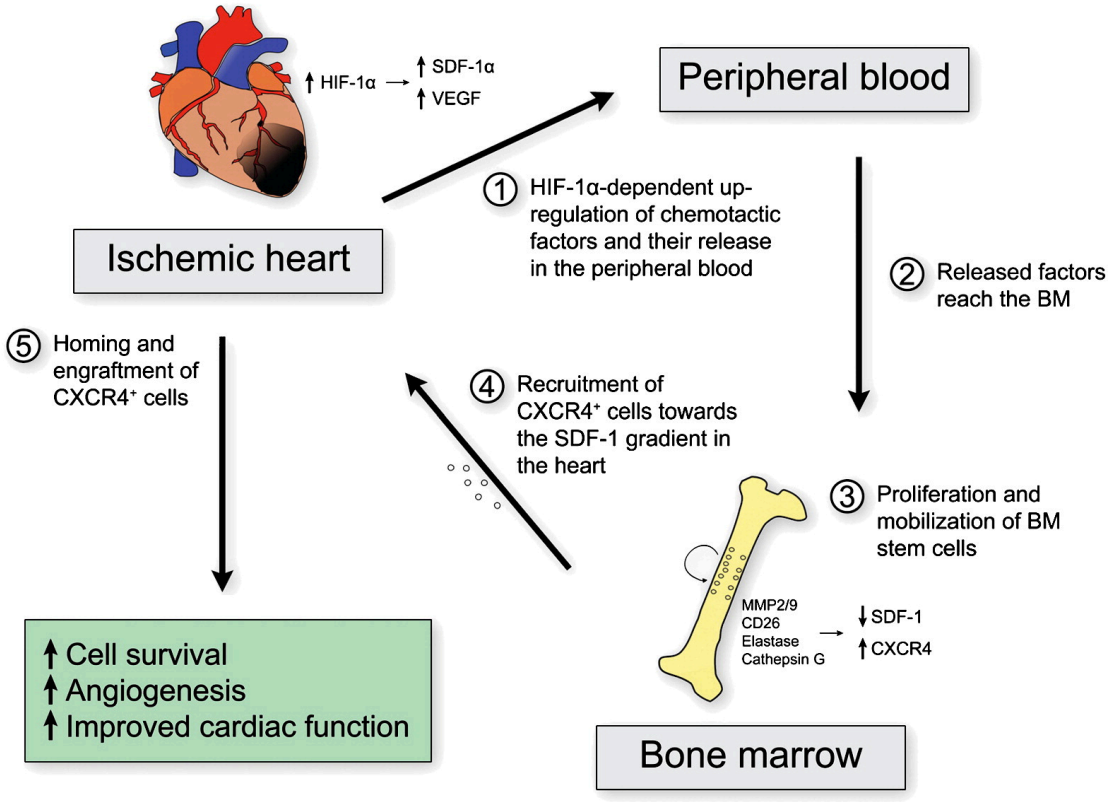


Figure 1.8 demonstrates the potential SDF-1α/CXCR4 receptor interaction and potential role in myocardial ischaemia. Myocardial ischaemia leads to the upregulation and release of the hypoxia signal, hypoxia inducible factor 1-alpha (HIF 1-α), which then upregulates and release stem cell mobilising signals such as SDF-1 α and vascular endothelial growth factor (VEGF). These signals reach the bone marrow resulting in the proliferation and increased expression of CXCR4 receptors of stem/progenitor cells (as well as degradation of SDF). This creates a change in bone marrow SDF gradient leading to the release of the stem/progenitor cells into the peripheral blood and towards the local SDF gradient originating in the myocardium. By homing into the damaged myocardium, they modulate myocardial repair and angiogenesis.

1.8.4 SDF-1/CXCR4: Therapeutic application in experimental models

Since the identification of SDF-1 as a homing factor, many experimental studies have taken place to target this particular axis to confer myocardial protection following an infarction. In particular, many of these studies have also confirmed the role of the SDF-1/CXCR4 axis in directing bone marrow derived stem cells to areas of infarcted tissue. Table 1.4 below summarises some of these experiments.

Animal Model	SDF-1 delivery method	Cells homed	Effects
Rat (241)	SDF-1 over-expressed fibroblasts + G-CSF	CD 117/34$^+$ Endothelial cells	Improved LV function, improved wall thickness and mass
Mouse (234)	SDF-1α over-expression (adenovirus)	Lin$^-$/GFP$^+$ BM cells	Increased stem cell homing
Rat (242)	Non viral SDF-1α transfection in skeletal myoblast	CD 117/34/31$^+$	Increased vessel density, improved LV EF/FS, increased posterior wall/septal thickness
Mouse (243)	SDF-1α infused into left ventricular cavity		Reduced infarct size
Mouse (244)	Local delivery of SDF-1α into ischaemic myocardium	GFP$^+$, vWF$^+$	Reduced infarct area, improved LVFS and vessel density
Rat (245)	Intra-myocardial delivery of protease resistant S-SDF-1 (S4V)	CXCR4$^+$ c-Kit$^+$	Improved capillary density/LVEF
Rat (246)	MSCs overexpressing SDF-1	GFP$^+$, α-actin SM cells, connexion 45$^+$ cells	Improved cell survival, LVFS and LV dimensions. Reduced apoptosis
Mouse (247)	Intramyocardial injection of SDF-1α		Reduced scar tissue, improved LVEF and neoangenesis. Cardioprotection (AKT)
Rat (248)	Intramyocardial delivery of SDF-1α/EPCs/cSA	CD31$^+$ BrdU$^+$	Improved LV/coronary flow, neovascularisation. Reduced inflammation.
Rat (249, 250)	Adenoviral mediated overexpression of SDF-1α (AdV-SDF-1)	c-Kit$^+$	Increased angiogenesis/anti-fibrosis/LVEF
Mouse (251)	Genetic/pharmacological inhibition of SDF-1 breakdown + G-CSF	CD45/CD34 /CXCR4$^+$, c-Kit$^+$, Sca-1$^+$	Improved cardiac remodelling/neovascularisation

Mouse (252)	Intramyocardial delivery of lentiviral engineered MSCc overexpressing SDF-1 α	GFP$^+$, BM cells, CXCR4$^+$, c-Kit$^+$, CD31$^+$	Improved LVEF, vascular density and reduced apoptosis

Table 1.4. Summary of experiments that have demonstrated the role of SDF in directing bone marrow stem cells to infarcted myocardium.

Furthermore, the same axis has been modified in experimental studies to further elucidate its role in MI. This can be illustrated by

- Delivering SDF-1α at the site of infarction whilst simultaneously inhibiting cleavage of SDF-1α
- Upregulating CXCR4 receptors in cardiomyocytes and cardiac stem cells
- Mobilising CXCR4$^+$ cells into peripheral circulation from bone marrow
- Upregulating CXCR4 in bone marrow stem cells

In a rabbit model of ischaemia-reperfusion by Misao et al (253), granulocyte-colony stimulating factor (G-CSF) administered following an MI was associated with an increase in SDF-1α level thus directing stem cells towards infarcted tissue; this led to a more favourable left ventricular ejection fraction, end diastolic dimensions and scar burden. This benefit was ameliorated in the presence of CXCR4 receptor blocker (AMD100) implying that the SDF-1 α/CXCR4 receptor interaction was central in mediating G-CSF mediated stem cell recruitment to the site of tissue infarction. Hu et al (243) on the other hand utilised in vitro and in vivo models of IRI to demonstrate the same axis; when pre-treated with SDF-1α, the degree of infarct size and apoptosis was reduced whilst there was an increased resistance to hypoxic cell injury via activation of ERK 1/2 and AKT. Saxenas group had demonstrated the preservation of cardiac function in a mice model of infarction when SDF-1α was administered into several peri-infarct zones. They too were able to observe increased phosphorylation of AKT together with upregulation VEGF in response to SDF-1α thereby providing potential mechanisms for the observed effects (247). Several authors have also demonstrated increased mobilisation of c-kit+ cells during adenoviral delivery of SDF-1α resulting in increased cardiac function

thought to be through the angiogenic and anti-fibrotic property of SDF (249, 250).

All these studies suggest SDF-1α mediated cardioprotection is due to the activation of downstream cell survival pathways and induction of neovascularisation rather than myocardial regeneration for which evidence remains incomplete (186). One limitation of using SDF-1α is the proteolytic degradation by exopeptidases thereby limiting its full therapeutic potential in myocardial ischaemia. To overcome this, researchers have bioengineered SDF-1α (S-SDF-1/SDV) to create a protein that retains its chemoattractant property whilst being resistant to proteolysis. Segers et al (245) were able to show that when this bioengineered version is injected into infarcted tissue, stem cells are recruited resulting in improved myocardial contractility. Using a similar concept, Zaruba and co-workers (251) used a 'novel therapeutic concept' of pharmacological and genetic inhibition of exopeptidases such as DPPIV in conjunction with G-CSF to recruit stem cells to infarcted myocardium in mice to demonstrate preservation of cardiac function through reduced cardiac remodelling and increased angiogenesis.

Many experimental studies have demonstrated chemokines, with SDF-1α in particular, being an important regulator in MI. I will therefore evaluate the effect of this chemokine in a human atrial trabecular model.

1.9 Stretch Preconditioning

The notion that the heart becomes dilated (stretched) during simulated ischaemia led authors to theorise that the beneficial effects of ischaemic preconditioning may be reproduced by mechanically stretching the heart (a term often referred to as 'stretch preconditioning') possibly through activation of mechanosensitive ion channels. Ovize and co-workers were probably one of the earlier investigators to demonstrate this. In their experimental model, stretch preconditioning was achieved in canine hearts by rapidly volume overloading the hearts prior to a period of simulated ischaemia. When compared to control hearts, the stretched heart significantly attenuated infarct size. This protection was abolished when $Gd3^{+}$, an inhibitor of stretch activated channel, was injected into the left atrium prior to stretching the heart. Intriguingly, the extend of myocardial necrosis remained unaffected (254).

In order to sought the mechanism, the same authors used a rabbit model to determine whether there were any cross talks between these mechanoreceptors and effectors of known preconditioning (K^{+}_{ATP} +/- adenosine receptors through activation of protein kinase C) and they were able to conclude this was indeed the case (107). Furthermore, isolated hearts stretched by a transient rise in left ventricular end diastolic pressure recovered better from an ischaemic insult when compared to their counter-part.

Using an isolated rat heart model, Obadia and colleagues were also able to demonstrate the existence of stretch-induced protection of the rat heart (255). Control group underwent 30 min of global ischaemia followed by 30 min of reperfusion. The intervening group underwent either (preconditioned group) 5 mins of global ischaemia followed by 10 min reperfusion or (stretch group) transient increase in LV preload from 5 to 20 cm H_20. In both intervening groups, there was reduced creatine kinase release, reduced incidence of ventricular fibrillation and enhanced post-ischaemic recovery of contractile function. In the intervening group, Staurosporine administration prior to either stretch or simulated ischaemia, abolished their beneficial effects suggesting the effects of stretch was mediated via protein kinase C (255).

Chapter 2: Aims and Hypotheses

2.1 General aims

The aims of this study were:

1. To confirm the ability to precondition human muscle using the atrial trabecular model of superfusion. Although this model has been established since the early 90's, it was important to confirm the ability to 'work the model'. This would allow the use of such model to investigate the protection of human muscle.

2. To characterise the model to achieve aim (1). This would be achieved by altering various components of the preconditioning protocol that include stabilisation time, duration of index hypoxia and reperfusion time. Established preconditioning protocols will be used and altered according to the ability to achieve successful preconditioning in human muscle.

3. To investigate whether stretching atrial trabeculae whilst reaching the peak of the Frank-Starling curve induces preconditioning. There is data from animal studies to support stretching as a preconditioning mimetic.

4. To investigate the ability for SDF-1α to chemically precondition human muscle. As discussed in the introductory section to this thesis, SDF-1α been shown in animal hearts to protect it from ischaemia-reperfusion injury via mechanisms analogous to other forms of conditioning.

5. To determine the presence of SDF-1α receptors on human cardiomyocytes and also to explore the mechanism involved in preconditioning human muscle using SDF-1α.

2.2 Hypothesis One

Human atrial trabeculae can be preconditioned using hypoxia and re-oxygenation.

This hypothesis was explored by:

1. Investigating whether a standard established preconditioning consisting of 3 mins hypoxia and 12 mins reperfusion preconditions human muscle. Further established protocols consisting of longer preconditioning hypoxia of 4 mins or longer reperfusion of either 16 mins were used.
2. Western blotting was used to confirm whether the stabilisation times used in this study was adequate. Specifically phosphorylation of Akt and Erk was investigated.
3. In establishing a protocol suitable for this study, various index hypoxia times were examined upon including 45, 60 and 90 minutes.

2.3 Hypothesis Two

Human atrial trabeculae can be chemically preconditioned using SDF-1α through the CXCR4 receptor.

This second hypothesis was investigated by:

1. Studying whether chemically preconditioning human muscle with SDF-1α protected the tissue from hypoxia-reoxygenation injury.
2. Determining whether protection involved CXCR4 receptor activation. Human muscle was pre-treated with AMD-3100 (SDF-1α receptor antagonist) prior to exposure to SDF-1α.
3. Confirming the presence of CXCR4 receptors on human cardiomyocyte using Western blot analyses.
4. Exploring whether Akt and Erk were mediators of the CXCR4 receptor activation by SDF-1α.

Chapter 3: General Methods

3.1 Introduction

Isolated human atrial trabeculae were used in the investigation into the protection of human muscle from hypoxia-reoxygenation injury. This superfusion model is described along with the Western blotting technique used. The use of the model within the Hatter Cardiovascular Institute has been described earlier (section 1.5.8.1).

3.2 Critical analysis of the superfusion model

This *ex-vivo* model is a unique robust and reproducible model for evaluating the cardioprotective potential of both pharmacological and non-pharmacological effects on the isolated human atrial trabeculae harvested from right atrial appendage.

This setup is a functional model that uses the developed force as a measure and recovery of function therefore is a surrogate marker of muscle injury. Data from this unique model has been reproduced with both positive controls using ischaemic preconditioning as well as negative controls of simulated ischaemia-reperfusion injury. The

effect of postconditioning has also been analysed using this model (256).

There are several advantages of this human model:

1. Avoidance of the confounding factor of collateral blood flow experienced with in-vivo models (127);
2. Eliminates the effects of alterations in preloads and afterloads. Authors have suggested in the past that brief period of ischaemia results in transient stretch (through dilatation) of the heart that may be responsible for preconditioning the human heart (254);
3. Eliminates any ethical considerations that may be involved whilst utilising experimental drugs;
4. In experiments involving whole hearts, altered cavity geometry occurs when there is a modulation of coronary perfusion pressure, tissue oedema and ischaemic contracture (as encountered during and following ischaemia). These may affect the measurement of contractile function (257) and
5. The natural milieu of the cardiomyocytes remains unaltered.

An important consideration of this model is that the human myocardium used is atrial rather than ventricular. This is based on

the relative ease of obtaining atrial tissue specimen and taking advantage of the fact that a significant proportion of cardiac surgery involves establishing cardio-pulmonary bypass. Importantly, there is data to suggest cellular models of ventricular myocytes can be preconditioned in a similar model to atrial trabeculae, therefore allowing data to be extrapolated (131). Similarly, in an atrial muscle model (where CK is released is measured as a marker of injury), preconditioning has been shown to occur (132). In current practice, it is now rare to resect ventricular tissue other than situations where aneurysmectomy is performed following a myocardial infarction or septal myomectomy in cases of hypertrophic obstructive cardiomyopathy. Utilising these samples on the atrial superfusion model would be difficult as resected tissue tends to be non-functional scar tissue (aneurysmectomy) or fragments of tissue that are not presented as a functional subunit (myomectomy) unlike atrial trabeculae that is almost a complete functional unit. Therefore all work conducted on human tissue in this thesis refer to human atrial muscle samples rather than ventricular specimens.

3.3 Oxygenation of human muscle

Delivery of adequate oxygen to all cardiomyocytes in an isolated trabecular model is determined by the oxygen demand of the cells (rate of oxygen utilisation), distance from the surface of tissue to its core in addition to the oxygen tension in which it is bathed (258). For these reasons if the oxygen demand is high or the diffusion distance from its surface-to-core is large, the isolated tissue (in this case human atrial trabeculae) may be ineffectively oxygenated even if the surrounding oxygen tension is high and therefore contractility will inevitably diminish. Therefore atrial trabeculae with small diameter that are adequately superfused with oxygenated buffer should in theory remain viable even without arterial perfusion. Furthermore for maximum contractility, these specimens must be small enough to enable diffusion of metabolic substrates (such as oxygen, glucose, pyruvate) and metabolites (lactate, carbon dioxide) at rates equal to each other but interestingly the diffusion of oxygen is the main determinant of contractility (258).

3.4 Appropriate diameter of trabeculae

Variability exists amongst published data with respect to what is considered the appropriate maximum diameter for effective oxygenation of superfused isolated muscle preparation. It was the pioneering work of Hill and colleagues who derived a formula based on 'biophysical principle' to determine the maximum diameter of superfused tissue that can be adequately oxygenated in an isolated muscle preparation (259). Using this formula, Prasad and colleagues were able to demonstrate the adequate oxygenation of isolated superfused human papillary muscle of diameter less than 0.932 mm in an atmosphere of 95% O_2 5% CO_2 with the temperature at 37^{o}C whilst being paced at 1 Hz (260). Page and colleagues on the other hand demonstrated that cylindrical cat papillary muscle with a diameter of 1.12 mm would be adequately oxygenated (261). In a separate experiment, Snow and colleagues demonstrated preservation of rabbit papillary muscle core with a diameter of 1.22 mm when it was paced at 0.8 Hz at 25^{o}C; however this will not be relevant to studies conducted here (262). In an interesting set of experiments conducted by Paradise and colleagues where rat papillary muscle diameter and oxygen tension were altered, they summarised that it would be only possible to adequately oxygenate

muscle with diameters that were less than 0.48 mm when oxygenated adequately at 37^{o}C and paced at 1 Hz (258). However it is well established from several experimental models utilising specimens obtained from human ventricle that is 1-2 mm muscle is adequate in superfusing isolated muscle (263, 264).

During this study, all human atrial trabeculae were equal to or less than 1.2 mm in diameter. When isolating the individual trabecula from the atrial appendage, the thinnest were isolated first. An assumption was made in that the human atrial appendages in this study were adequately oxygenated and majority of the studies above would advocate this. If the diameter of the muscle were such that core hypoxia existed, then this would affect all groups equally. The results obtained in the experiment would remain valid since the study compares the responses of clusters of trabeculae that were of equal characteristics at baseline.

Based on the above and experiences of previous researchers using this model, a cut off value of 1.2 mm was arrived. On the principles of diffusion, one would expect atrial trabeculae greater than 1.2 mm in diameters not react in the same way to simulated ischaemia-reperfusion or hypoxic preconditioning when compared to tissue of smaller diameter. However, there is evidence from models using

ventricular papillary muscle obtained from explanted heart that are 1-2 mm in diameter that have been successfully preconditioned (129, 263, 264). With the notion that smaller diameter trabeculae may be better diffused with gases and solutes, all trabeculae with diameters up to 1.2 mm have been included and their baseline function value are similar without any statistical difference between groups.

Finally, there is most likely to be a core of necrosis in this experimental model. Contraction is most likely due to an outer layer of viable cardiomyocytes. Following a long experimental protocol of at least 180 minutes, it is likely that the numbers of viable cells are reduced even further. This precludes further experimentation at the end of the protocol (such as Western blot analysis). Therefore this atrial trabecular model demonstrates indirect evidence through a functional recovery model without any exhibition of mechanistic pathways as demonstrated in cellular mechanisms of preconditioning.

Despite the above limitations, this experimental model is unique, has several advantages and is reproducible by different researcher albeit different protocols (108, 133, 134, 136, 265, 266). Despite the initial model of preconditioning was based on a different model (74), this translational model enables us to determine whether data obtained

from animal studies can be applied to human models therefore allowing us to better our understanding of preconditioning and study the effect of novel proteins such as SDF-1α may have on myocardial injury.

3.5 Study subjects

Patients recruited in this study were undergoing elective coronary artery bypass graft (CABG) surgery, valve repair or replacement surgery and replacement of the aortic arch at the Heart Hospital, University College London NHS Foundation Trust, London. All patients recruited had given prior written consent using the consent form as shown (see appendix).

Ethical approval for this particular study was obtained from the Research and Ethics Committee (REC), sub-committee alpha, (REC no. 00/00275) at the University College London/University College London Hospitals, in line with the Medicines and Healthcare products Regulatory Agency (MHRA), United Kingdom. Consent was obtained following admission to hospital either on the evening before or morning of the surgery prior to receiving any premedication that may influence their decision-making ability. Given the nature of the

study it was difficult to attend outpatient clinics prior to surgery for recruitment purposes.

3.5.1 Exclusion criteria

Patients were excluded if:

- They were aged above 80 years
- Suffered with unstable angina 72 hours prior to surgery
- Experienced a troponin positive event within 6 weeks of undergoing surgery
- They had a history of arrhythmias e.g. atrial fibrillation or flutter, ventricular arrhythmias within 6 weeks or on any anti-arrhythmic medications such as amiodarone
- They had any forms of heart failure (ejection fraction less than 50%) or active pulmonary oedema
- Had established impaired renal function (defined by a creatine level above the reference value provided by University College Hospitals NHS Foundation Trust biochemistry laboratories)
- Patient had any form of diabetes – it is known that this subset of patients requires a larger preconditioning stimulus (267).
- They were unable to provide a valid consent

3.6 Human atrial trabecula model of simulated hypoxia-reperfusion injury

3.6.1 Specimen collection and transport

During the surgery, prior to insertion of the venous cannula (usually follows arterial cannulation of the aorta), a purse string suture would be placed around the base of the right atrial appendage to enable harvesting of the sample. At this point, the surgeon would be aware to manipulate the appendage with caution as rigid or excessive handling of the tissue would quite often affect the functionality of the trabeculae therefore invalidate any experiments. A clamp (side biter) would then be placed above the purse string suture and a scissor would be used to carefully dissect out the right atrial appendage by the cardiothoracic surgeon. The sample would then be placed immediately in a 50 mL Falcon™ tube containing approximately 45 mL of modified Tyrode's buffer (comprising in mmol/L 118.5 NaCl, 4.8 KCl, 24.8 $NaHCO_3$, 1.2 KH_2PO_4, 1.44 $M_gSO_4.7H_2O$, 1.8 $CaCl_2.2H_2O$, 10.0 glucose and 10.0 pyruvic acid), which was pre-oxygenated with a gas mixture consisting of 95% O_2 + 5% CO_2 to maintain a physiological pH between 7.35-7.45 and a partial pressure of oxygen and carbon dioxide above 55 kPa and 4.0-6.0 kPa respectively. Temperature was maintained <4°C by placing

the Falcon™ in a flask containing ice which was then transported back to the laboratory as soon as possible.

On the morning of the experiment, the organ bath system would be set-up with the water jackets switched on and the buffer circulating to eliminate any delays in conducting the experiment on return from the Heart Hospital. In a separate polystyrene box containing ice, a 50 ml Falcon™ tube containing the modified Tyrode's solution would be bubbled with 95% O_2 + 5% CO_2 gas mixture for a minimum of 5 minutes prior to placement in a transport flask containing ice. On arrival to the Heart Hospital, the flask containing the Tyrode's solution would be placed immediately into a 4°C fridge next to the operating theatre. The flask would then be removed from the fridge within minutes of the surgeon harvesting the right atrial appendage. This enabled the temperature to be regulated at the preferred 4°C. The transport between the Hatter Cardiovascular Institute and Heart Hospital was approximately 1.1mile journey; walking would take approximately 20 minutes whereas cycling would take 10 minutes. At the set-up of the experiments, samples were transported on foot but later a pushbike was utilised to minimise transport times to maximise yield in terms of surviving trabeculae.

The importance of temperature regulation and transport times has been highlighted, as these are some of the variable factors that influence whether or not trabeculae survived.

3.6.2 Dissecting atrial trabeculae

Once in the laboratory, the sample would be placed onto a clean petri dish containing a gel base to which the specimen would be immobilised against using pins with the inner surface exposed (see figure 2.1)

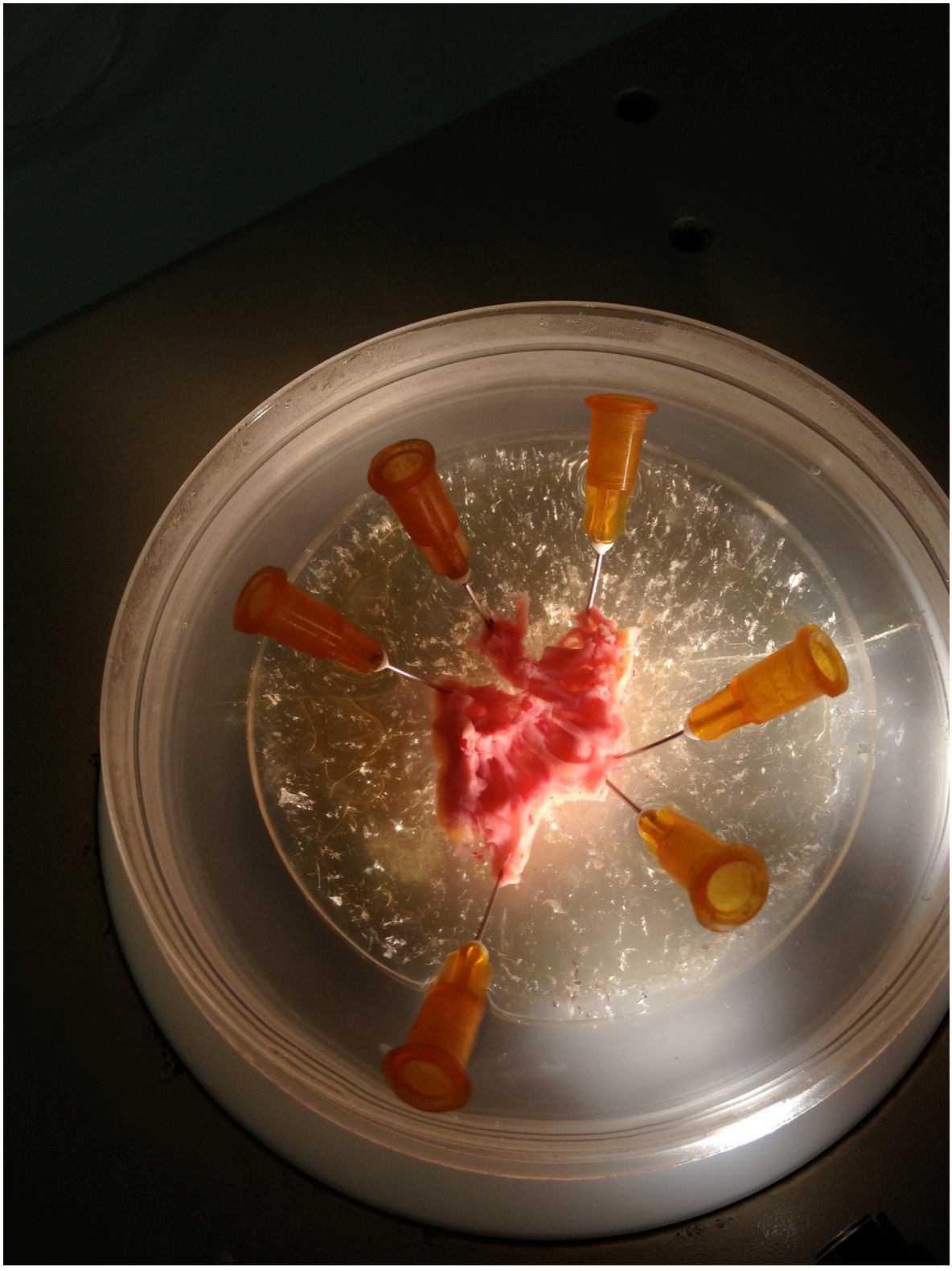


Figure 3.1. Atrial appendage fixed onto a gel base with the inner surface exposing multiple atrial trabeculae.

The dish itself contained modified Tyrode's buffer that was continuously oxygenated with 95% O_2 + 5% CO_2 gas mixture and placed on a bed of ice to maintain temperature of 4°C. The edges of the inner surface of the atrial appendage were secured onto the gel bed to enable exposure of the atrial trabeculae that were dissected out. Individual trabecula (>2mm length, <1.2mm diameter) were identified under a light microscope (Olympus SZ40) using a separate light source (Photonic PL2000) then isolated by tying a surgical knot at either end of the trabecula with a 7-0 silk suture. Using a microscope facilitated enhanced identification of trabeculae, minimised manual manipulation and also allowed measurement of trabeculae prior to their dissection. Once excised, the trabeculae were suspended vertically in a 25ml water-jacketed organ bath between two platinum pacing electrodes (Radnoti Glass Technologies, USA) containing modified Tyrode's buffer bubbled with a gas mixture containing 95% O_2 + 5% CO_2 to maintain a physiological pH between 7.35-7.45 and maintain pO_2 > 55 kPa and CO_2 between 4.0-6.0 kPa. The inferior end of the trabecula was attached to a fixed post in the organ bath using a 7-0 silk suture and the opposite superior end was attached to a force transducer also using a 7-0 silk suture. Where there were multiple trabeculae

isolated from a single atrial appendage, each trabecula would be placed immediately into the organ bath attached to the pressure transducer only and dissection would continue so that all trabeculae were dissected out. It took an average of 10 minutes to dissect out each trabecula and the beginning of the experiment would be taken at the point when the opposite end was attached to the fixed glass post and the initial stretch of 0.1g was applied. The entire apparatus was placed on a low vibration table. A heat exchanger was used to maintain temperature at 37°C (Thermo Electron Corporation, USA). There were a total of 4 baths for use in any one experiment.

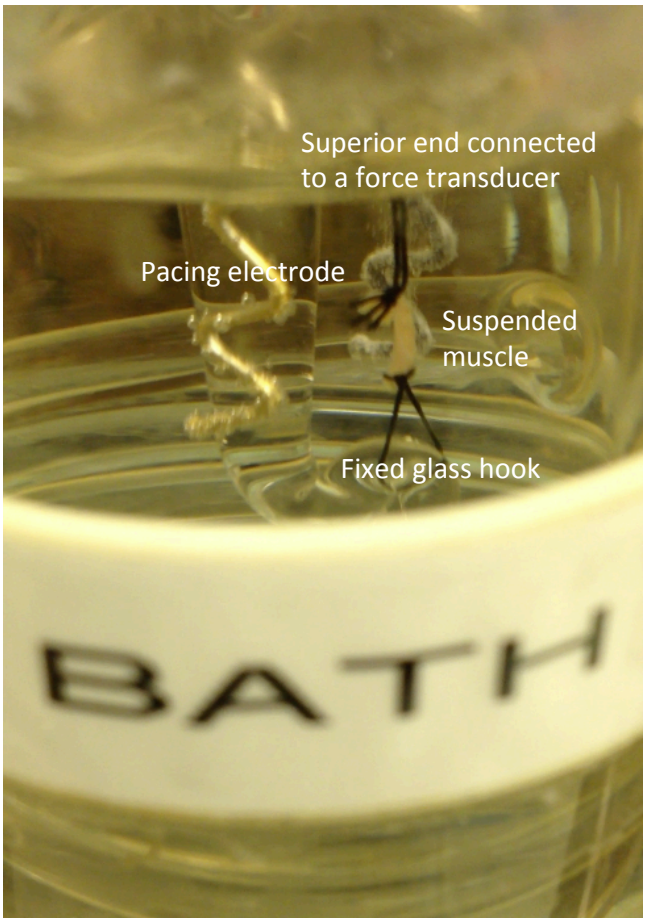


Figure 3.2. A single trabecula suspended in an organ bath

Tyrode's solution was used in these superfusion experimental models due to its isotonic physiological property to human tissue. It has been modified from Ringers lactate solution and also differs from other experimental mediums used in animal studies including Krebs Heinseleit solution (comprising in mmol/L 118.5 NaCl, 4.8 KCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $M_gSO_{4.}7H_2O$, 1.7 $CaCl_2.2H_2O$, and 11.0 glucose). Modified Tyrode's buffer contains pyruvate in addition to other standard components; pyruvate serves to act as an immediate source of energy on entering the Krebs cycle to produce adenosine tri-phosphate (ATP), whereas glucose would normally be required to be broken down via the glycolytic pathway to produce pyruvate.

3.6.3 Set-up of the basic model: Simulation of hypoxia and re-oxygenation

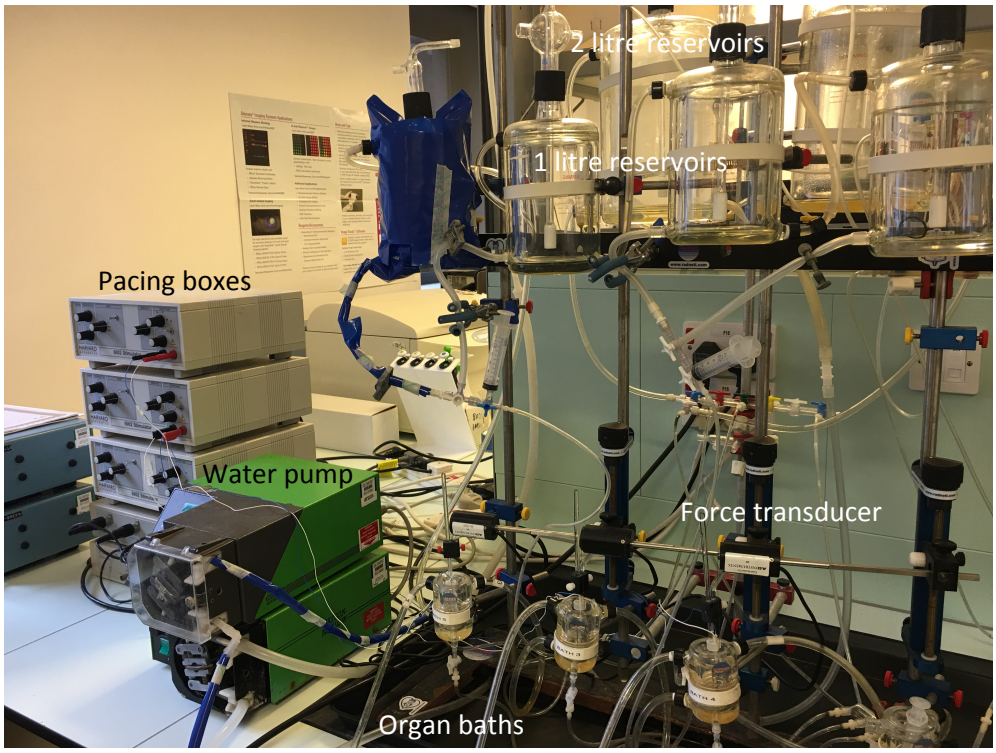


Figure 3.3. Setup of the experimental model.

The suspended trabeculae in non-toxic perspex 25 ml **organ baths** were continuously bathed in modified Tyrode's buffer delivered from **2 litre reservoirs** that was bubbled with gas mixture consisting 95% O_2 + 5% CO_2 at 37°C. The delivery of the buffer was via tubing made up of either silicone or polythene. The flow itself was regulated using adjustable clamps attached to the tubing itself. The buffer would flow into the organ baths and the effluent would be collected in a

large glass beaker that would be re-circulated using a **peristaltic pump** (Watson-Marlow 502S, Cornwall, England). This constant replacement of buffer ensured the sample was continuously supplied with fresh nutrients. The pH, pO_2, pCO_2 and $HCO3^-$ were observed through frequent sampling of the effluent using an automated blood gas analyser (AVL 993, AVL Medical Instruments, Switzerland) to ensure the pH was kept physiological between 7.35-7.45. The temperature, monitored by a thermocouple, was maintained at 37°C, by circulating warm water through the water jacket. During simulated ischaemia, the superfusate that was free of substrate, was bubbled with 95%N_2 + 5% CO_2 allowing it to become hypoxic (pH 7.24-7.34). Once the trabeculae were suspended, they were **paced** by field stimulation at 1 Hz under normal conditions and 3 Hz during simulated ischaemia. The platinum pacing electrodes were attached to a stimulator (Harvard Apparatus) that delivered the pacing voltage. Threshold stimulus was specific for each trabecula and would range anywhere between 10-30 volts. The force transducer was connected to a multi-channel quadbridge amplifier (AD Instruments) and using the PowerLab (AD instruments) converted the motion from the transducer into interpretable spikes on the software application (Chart 5 for Windows™). At the end of each

experiment, the length and width of trabeculae were measured with an eyepiece graticule in the operating microscope.

3.6.4 Calibration of temperature and flow of modified Tyrode's buffer

The water bath was set at 41°C to achieve a temperature of 37±0.1°C allowing for a degree of insensible loss to the environment. Although this was the case for most experiments, depending on the laboratory temperature this would vary and was adjusted accordingly.

The flow of Tyrode's solution into the organ bath was adjusted to ensure the rate of flow was kept constant at 180ml/hour to maintain an equilibrium (rate of into organ bath = rate out of organ bath). This was achieved by adjusting the revolutions of the pump in conjunction with side clamps attached to the tubing.

3.7 Basic experimental protocol

3.7.1 Stabilisation phase

Trabeculae were suspended vertically in the organ bath as described above. The initial phase was the stabilisation phase followed by simulated ischaemia then ending with reoxygenation (simulated reperfusion). The duration of individual component varied throughout my research period. Through characterisation of the human trabecular model, the optimum timing of the individual components was determined which was then applied to the rest of the experiments conducted. This is discussed below.

With the muscle suspended and paced at 1 Hz at 37°C, the force transducer would be reset to zero. A small stretch would be applied to the muscle to create a resting muscle tension of 0.1g and the muscle would be left for 30 minutes. A sample of the effluent would be taken to ensure the pH, O_2, CO_2 and $HCO3^-$ were within normal physiological levels. A good healthy sample would be seen contracting immediately and this would improve over time prior to application of various experimental protocols. A surrogate marker of the quality of specimen was noted by also recording the rate in

change in force (dF/dt) for each set of experiment conducted; the smaller dF/dt, the stiffer the muscle and poorer the quality.

The muscle was gradually stretched using a micromanipulator in a stepwise manner over a period of 15 minutes until maximum force of contraction was achieved through the Frank-Starling law of the heart.

The law states that the stroke volume of the heart (developed force of contraction) increases in response to an increase in the volume of blood returning to the ventricle (stretch), when all other factors remain constant. With each stretch, the specimen would lengthen. This in turn would lead to an increase in resting tension that would gradually return to baseline. A minimum of 0.1g of force would be applied every minute and allowed to plateau before a further stretch was applied. During each experiment a graph would be plotted with resting tension on the X-axis and developed force on the Y-axis. Once the peak of the Frank-Starling curve was reached, the force applied to the muscle would be reduced to the resting force just prior to reaching the peak. This ensured that muscle was not overstretched.

All muscles were then allowed to equilibrate for at least 45 minutes.

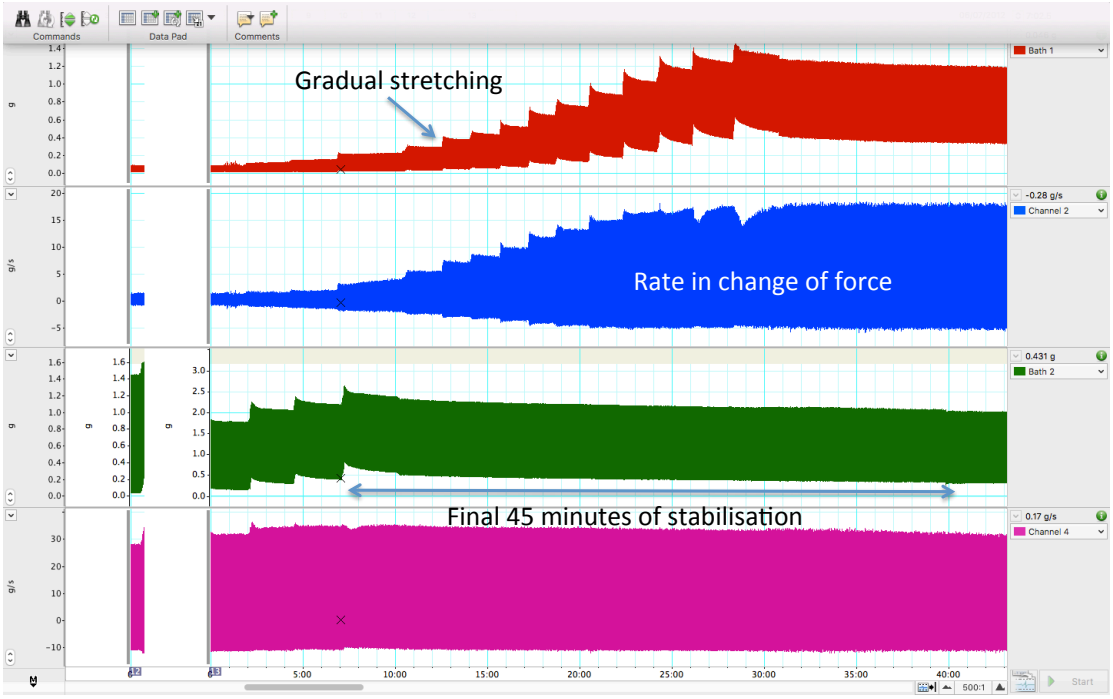


Fig 3.4. Demonstrating response of human muscle to stretch and final stabilisation phase.

The top recording (red) demonstrates that with each stepwise increase in muscle length, the developed force is increased. This is continued until the peak of the Frank-Starling curve is reached.

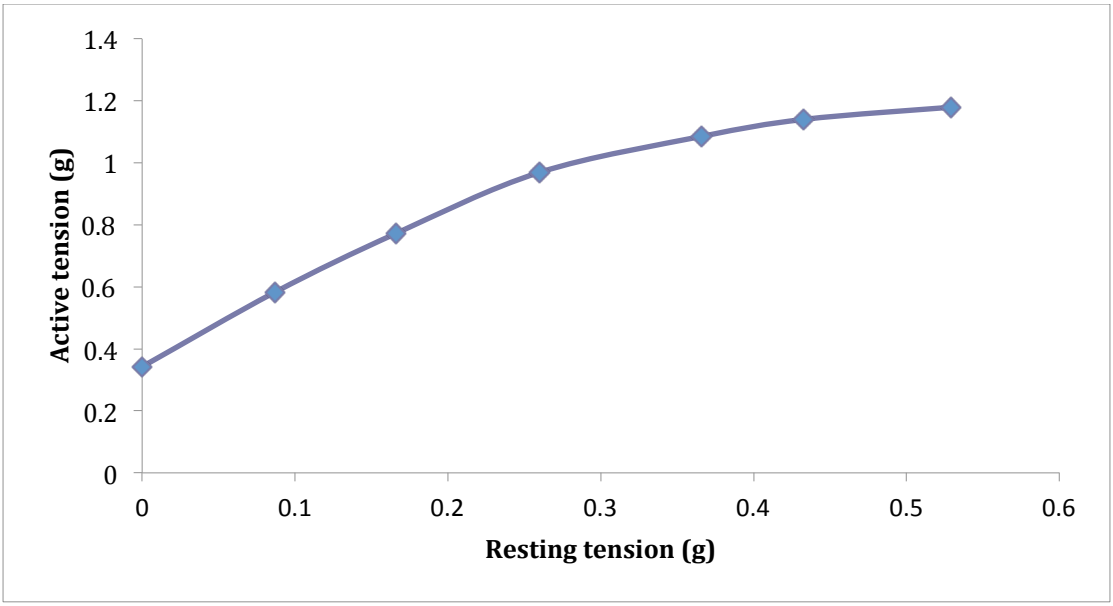


Figure 3.5. Graph taken from an actual experiment demonstrating a typical Frank-Starling behavior of human atrial trabeculae.

Stretching the muscle with a micromanipulator increases the resting tension (X axis), which in turn increases the active tension (Y axis). Towards the end a plateau is reached indicating that the peak of the Frank-Starling curve has been achieved.

3.7.2 Simulated ischaemia and reperfusion

Following the stabilisation phase, the tissue would be subjected to a period of simulated ischaemia.

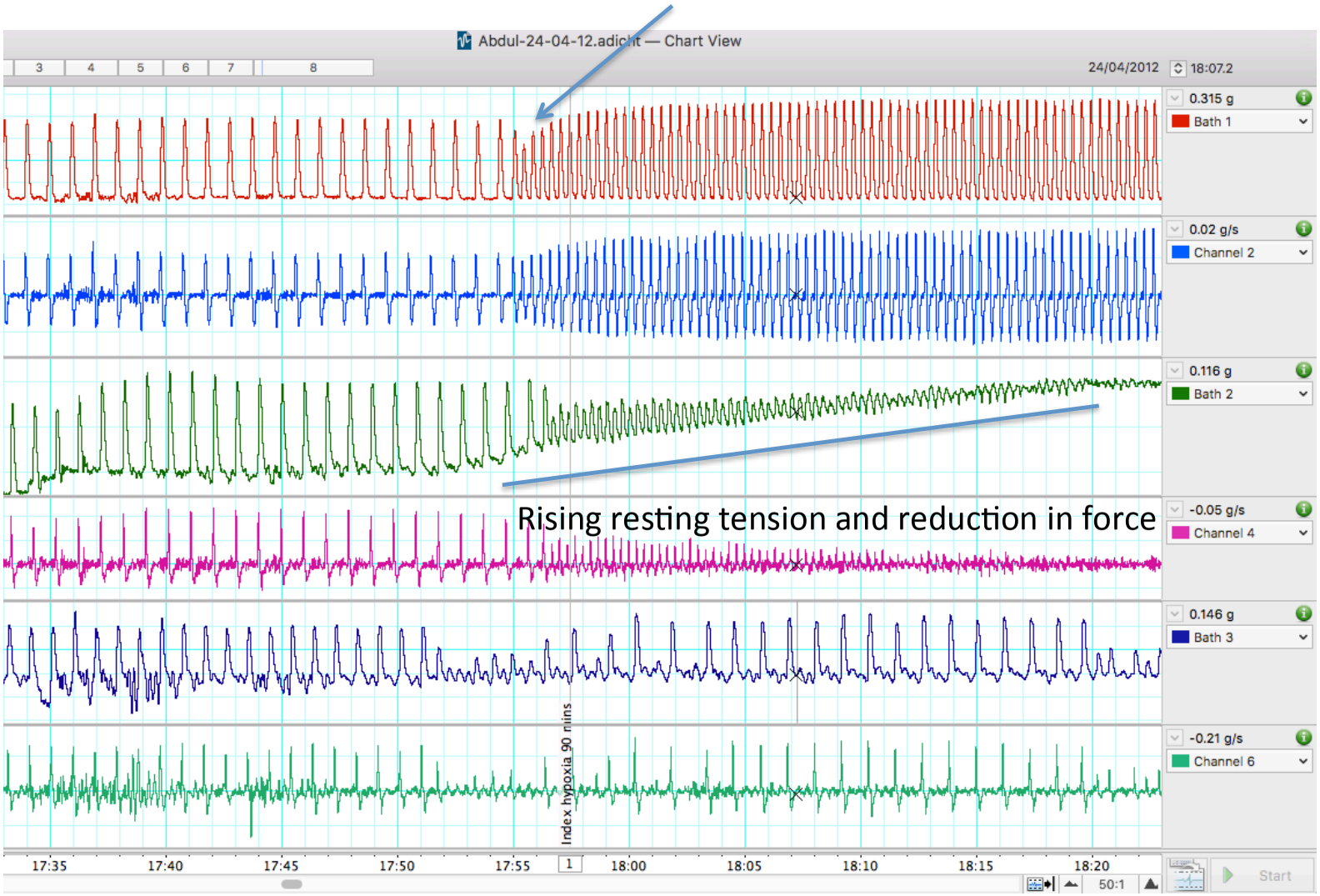
All the water-jacketed organ baths were arranged in series with the overflow of effluent collecting in a large glass beaker that was re-circulated back into the 2-litre chambers. There were two 2-litre

chambers containing normoxic and hypoxic buffers. Three way taps controlled the flow to each organ bath. There were also smaller 1-litre chambers connected to individual baths allowing a different agent to be tested on a particular trabecula. For example, at any one time there could be a sham experiment running, alongside a positive control (hypoxic preconditioning), negative control (simulated ischaemia-reperfusion) and one being chemically preconditioned (SDF-1α). Each organ bath itself was connected to a drain controlled by a valve that enabled rapid replacement by a different buffer.

Ischaemia for at least 60 minutes was simulated by replacing the oxygenated glucose and pyruvate-containing Tyrode's buffer with a hypoxic pyruvate-free and glucose-free buffer (comprising in mmol/L 118.5 NaCl, 4.8 KCl, 24.8 $NaHCO_3$, 1.2 KH_2PO_4, 1.44 $M_gSO_4.7H_2O$, 1.8 $CaCl_2.2H_2O$, 7.0 choline chloride) that was bubbled with 95% N_2 + 5% CO_2. Field stimulation was increased from 1 Hz to 3 Hz to enable depletion of ATP levels. Oxygen tension was maintained between 6-8 KPa whilst the pH was maintained at a lower level between 7.30-7.35. The induction of simulated ischaemia was immediately apparent on the recording chart, as indicated by a reduction in force amplitude and increase in contractile frequency. During this period the resting tension would also rise up to the point

of reperfusion at which point the hypoxic buffer would be replaced by normoxic buffer and the field stimulation would be reduced to 1 Hz. During reoxygenation (simulated reperfusion), the resting tension would also begin to return near baseline. This is shown in figure 3.6 below which is a snapshot taken from an actual experiment.

Point of hypoxia and increase in pacing frequency to 3 Hz



3.8 Measurement of force

As described earlier, the trabeculae were suspended vertically in modified Tyrodes buffer in a 25 ml perspex organ bath between a force transducer and a fixed hook and paced by field stimulation by platinum pacing electrodes that were on either side of the trabeculae. The pacing was driven by an isolated stimulator (Harvard apparatus) triggered by a computerised clock. The amplitude (voltage) and duration (pulse width) of the square wave pulse could be altered by adjusting the appropriate dial on the stimulator. The pulse width for all experiments were set at 5ms and amplitude was set at least twice the threshold needed by individual trabeculae for mechanical contraction to take place. Through the force transducer, amplifier and PowerLab software, spikes were seen which represented the developed force. Prior to the start of each experiment, the entire system would be reset. In particular, a known weight would be applied to the force transducer to ensure that the appropriate developed force was being recorded (calibration of force transducer). At the end of each experiment, the entire system would be drained and circulated with distilled water before being flushed. Every 3 months, the system would undergo an acid wash as recommended by the manufacturers.

3.9 Application of protocols and infusion of compounds

Through this system, various protocols could be applied. This would include different stabilisation, simulated ischaemia and reperfusion times. Similarly a range of protocols of ischaemic preconditioning could be applied by alternating between the oxygenated normoxic buffers or oxygen and substrate deplete hypoxic buffer. Various compounds could also be investigated upon through separate infusion from 1-litre chambers that were connected individual 25ml organ baths. Recirculation of effluent was via a separate pump that collected the effluent and re-circulated back into these 1-litre chambers.

A randomisation technique was applied to the experiments to eliminate any bias. The dissected trabeculae were allocated to a bath depending on the outcome of a shuffled pack of cards numbered one through to four. The bath in turn had a predetermined protocol applied to it at the beginning of the experiment.

3.10 Measure of cardioprotection: Recovery of function

The developed force of contraction was recorded on an Excel spreadsheet at 15-minute time intervals throughout the experiment

by a built in programme within Lab Chart. Baseline force at *t=0* was taken at 1 minute prior to subjecting tissue to simulated ischaemia to allow for any error that may arise whilst switching between buffers. This represented the maximum contractility of the trabeculae. At the end of the protocol, the developed force was also noted and this was divided by the baseline value to calculate the recovery of function, which was expressed as a percentage of the baseline.

3.11 Measure of stability

During various stages of the experiment there was a group of atrial trabeculae that would be allowed to contract for the entire duration of the experiment (minimum 210-minutes) in one of the organ baths, superfused with oxygenated modified Tyrode's solution and paced at 1Hz with no further experimental manipulation following the initial stabilisation phase. This was to ensure that the atrial trabeculae were stable and more importantly provided reassurance that the organ bath system was in good working order.

Over time there was a gradual decline in function over the duration of the experimental protocol. Encouragingly, this compares favourably with the decline in contractility observed in other atrial

trabeculae studies. Using a comparable experimental model, Böhm and colleagues observed a 20% reduction in function over 3 hours where the atrial sample was continuously bathed in oxygenated solution but changed intermittently and paced at 1 Hz. Other examples include work from researcher carried out in Yellon's laboratory that noted a reduction in function by 36% over a time period between 2-4 hours.

3.12 Exclusion criteria

Trabeculae were excluded from the study:

- The trabeculae had to be of a certain 'quality' to be included in the study. Those that failed to reach a baseline contractility of 0.5g were excluded. This was determined based on experiences from previous researchers at The Hatter Cardiovascular Institute
- Damaged trabeculae were excluded. This may have occurred during the harvesting process where part of the trabeculae would be excised or during the isolation process itself in the laboratory. Any trabeculae that needed separating from other trabeculae would also be excluded as the dissecting process would inevitably damage the muscle

- Trabeculae greater than 1.2mm thickness were excluded based on the principle of adequate oxygenation and diffusion of oxygen to muscle core
- Any irregularly contracting trabeculae either at baseline or towards the end of protocol.

3.13 Western Blot analyses

My gratitude is to Dr Andrew Hall, postdoctoral research fellow who kindly taught and assisted me during this part of my research.

This widely used analytical method detects specific proteins from a sample of homogenate. During this technique, proteins are separated based on their type and molecular weight through gel electrophoresis. This is then transferred to a membrane that produces a band for each protein. This membrane is then incubated with synthetic antibodies that bind with these specific proteins of interest. The unbound antibodies are then washed off leaving behind the bound antibody of interest that is detected by developing the film. The thickness of the band corresponds to the amount of protein present (268).

As part of the characterisation, Western blot analysis was performed from samples obtained from patients as described earlier. Harvested right atrial appendages from patients undergoing cardiac surgery were placed in a 50ml Falcon tube containing at least 45ml pre-oxygenated normoxic Tyrodes buffer at 4°C and transported to the laboratory where the appendage was divided into five and placed in separate organ baths. The purpose of Western blotting was to determine the optimum stabilisation time prior to any experimental intervention. The samples were kept in the bath for a predetermined duration (as though they were being stabilised as part of an experimental protocol) then snap frozen in liquid nitrogen prior to placement in a -80°C freezer. Western blot analysis was then performed at a later day after several specimens were collected. The method is described below.

In bath 1 the sample was kept for 10 minutes, bath 2 for 30 minutes, bath 3 for 60 minutes, bath 4 for 60 minutes with insulin added at 45 minutes and bath 5 for 120 minutes.

3.13.1 Protein extraction

The specimen was suspended in a 10% phosphate buffered saline (consisting in mmol/L NaCl 100, TRIS 10, EDTA 1, sodium pyrophosphate 2, sodium fluoride 2, β-glycerophosphate 2 with 4-[2-aminoethyl] benzenesulfonylfluoride hydrochloride 0.1mg/ml, pH 7.4, and Sigma protease inhibitor cocktail) in a 15ml Falcon™ tube placed on a bed of ice to maintain temperature at around 4°C. The lytic agents enabled extraction of protein from the cells whilst the protease inhibitors prevented degradation of the proteins. The sample was then homogenized at 24 000 revolutions per minute using a Polytron T25 homogeniser (IKA Labortechnik T25, Janke & Kunel GmbH & Co, Germany). The homogenate then underwent certifugation at 10,000 x g for 10 minutes at around 4°C. The supernatant deep to the layer of fat was aspirated and stored in 160µl aliquots. Further supernatant were drawn off to quantify the amount of protein so that calibration could take place to calculate the amount of protein required for gel preparation.

3.13.2 Protein quantification

Protein quantification was determined using the detergent-compatible Pierce Bicinchoninic acid (BCA™) based assay reagent system (Rockford, USA). This colorimetric assay reduces ionized copper to produce a purple coloured BCA-copper complex; the quantity produced is proportional to the protein content. A spectrophotometer set at a wavelength of 562nm was used to estimate the protein content. A standard curve using increasing concentrations of bovine serum albumin (BSA) was produced (0, 10, 20, 30, 40 µL of 2µg/ml and 25µL of 4µg/ml). The relationship between light absorbance and protein concentration was linear ranging from 20-2000 µg/ml. The protein concentration in each specimen could therefore be obtained by plotting the absorbance on a standard curve. This made certain that there was equal loading of the polyacrylamide gel.

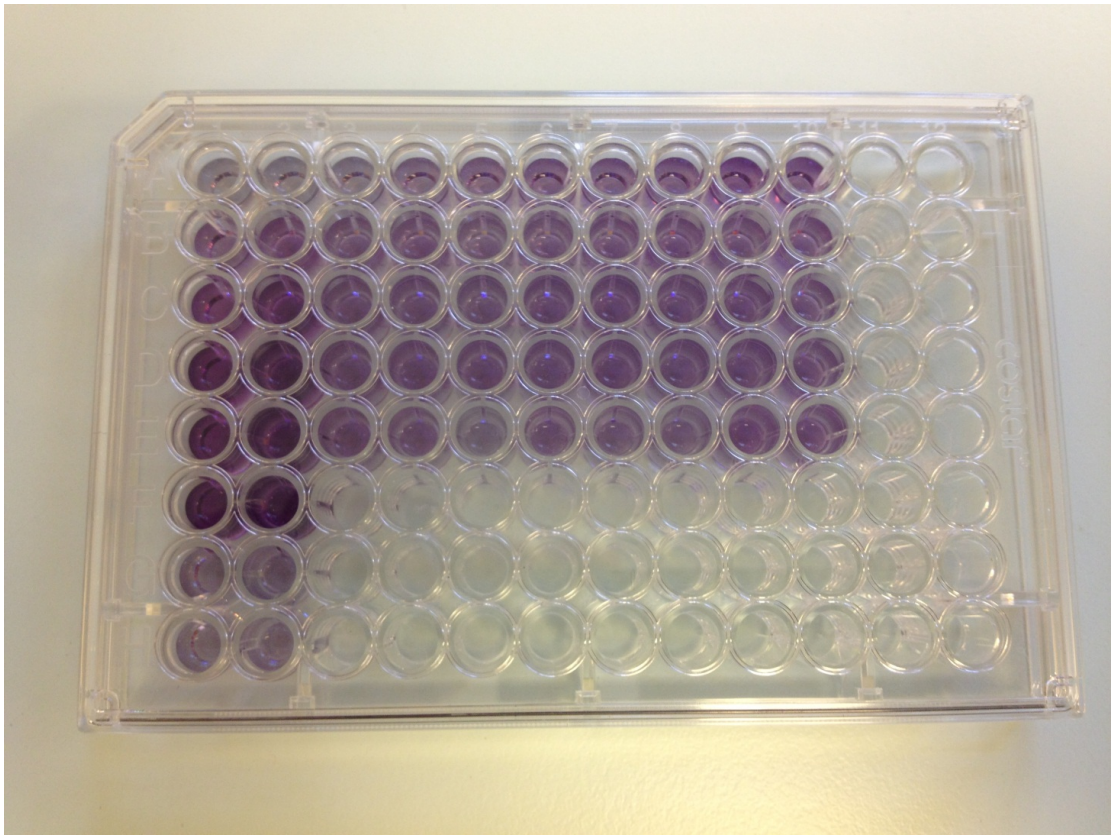


Figure 3.7. Actual plate layout demonstrating BCA-copper complex ($Cu2SO_4$).

3.13.3 Gel preparation

Sodium dodecylsulphate-polyacrylamide gels were prepared (SDS-PAGE). Gel mixtures were introduced between two glass plates that were prepared by cleaning them with 70% ethanol. The plates were separated by spacers loaded onto a gel loading self-sealing system. The lower proportion of the SDS-PAGE gel was a 12.5% running gel that was essentially one large sized gel (12ml distilled water, 9ml

running gel base [containing 1.5M TRIS, 0.4% SDS in distilled water, adjusted with HCl to a pH of 8.8], 15ml of 30% acrylamide and mixed prior to the addition of 40µL TEMED, 200µL 10% ammonium persulphate (269)). Once the running gel was allowed to set for 30 minutes an upper 5% stacking gel (7ml distilled water, 3ml of stacking gel base [0.5M TRIS, 0.4% SDS in distilled water, adjusted with HCl to a pH of 6.82], 2ml of 30% acrylamide, 20µL 8% bromophenol blue, 24µL TEMED and 120µL 10% APS) was prepared. Wells were formed in the upper stacking gel by inserting a 24-pronged comb between the glass plates at the top prior to introduction of the stacking gel. This was allowed to set for 10 minutes.

3.13.4 Electrophoresis technique

The gel plates were mounted onto a water-cooled electrophoresis system. Running buffer (comprising of 1L-distilled water, TRIS 3.03 mmol/L, SDS 1.0g/L and glycine 14.42 g/L) was added to both ends of the plates ensuring that the electrodes were covered. A dual colour molecular weight marker (BioRad, UK) was pipetted into the first well followed by 45 µg of protein sample (prepared as described

in protein extraction) into each subsequent well. The gels were then allowed to run at 120 volts constantly until the protein marker showed good separation (usually 2-4 hours).

3.13.5 Protein transfer

Following electrophoresis, gels were removed, cut to size and placed on Whatmann filter paper that was then rolled to ensure any air bubbles were eliminated. A nitrocellulose membrane (Hybond ECL, Amersham, UK) was also trimmed to the same size as the gel and placed on top and rolled again. A second Whatmann filter paper was placed on top of the nitrocellulose membrane and gently flattened to expel any trapped air bubbles. This whole sandwich was then mounted on a transfer cassette and placed vertically in a transfer buffer containing transfer tank (transfer buffer consisted of 700ml distilled water, 200ml methanol, 100ml of 10 times strength transfer buffer stock solution [comprising 1L distilled water, glycine 144.2 g/L and TRIS 30.3 g/L]). The transfer system was run at 0.1 mA overnight for a minimum duration of 16 hours. On the following day, the membranes were removed and stained with Ponceau Red (Sigma

Chemicals, Poole, UK) to ensure adequate transfer of proteins from the gel.

3.13.6 Immunoblotting

The membranes were placed on trays with a rocking platform and washed with TBS-Tween (comprising 1L distilled water adjusted to pH 7.6 with HCl, NaCl 8.0g, TRIS 2.42g, Tween-20 [polysorbate-20] 1 ml) for 5 minutes. Subsequently a wash in blocking buffer (5g Marvel – dried skimmed milk powder in TBS-Tween) was performed for one hour to minimise interference from non-specific binding of antibodies to other proteins. Three further 5-minute washes were performed with washing buffer following which the membrane was probed for a minimum of 2 hours with the primary antibody being analysed. Dilutions of 1:1000 were used for the phosphorylated and total kinase antibodies. The antibodies were diluted in a 5% BSA solution. Three further 5-minute washes were performed in TBS-Tween prior to probing with the secondary antibody for a minimum of 1 hour (same dilution as their primary antibody) using blocking buffer as a diluent. A final three 5-minute washes were performed prior to the membranes being incubated with enhanced

chemiluminescent (ECL) Western blotting detection reagent (GE Healthcare, UK) to produce the horseradish peroxidase signal.

3.13.7 Protein band quantification

Within a dark developing environment, the nitrocellulose membranes were exposed against Amersham Hyperfilm ECL and subsequently developed using Kodax GBX developer and fixer (Sigma chemicals, Poole, UK). Once the films were dried, they were digitally scanned and stored for analysis. Image J (National Institute of Health, USA) was used to obtain densitometry readings of the bands. Values of absolute phosphorylation of proteins were obtained along with values normalized against the total protein.

3.14 Immunohistochemistry for SDF receptor and mechanism

My gratitude is to Dr Jose Vicencio, postdoctoral research fellow who kindly conducted this part of my research on atrial trabeculae which I provided and the method described below is as described in our published paper (270).

In this group of experiments, isolated human atrial trabeculae were frozen and mounted in optimal cutting temperature (OCT) before being cut into 5 μm sections at −20°C in a microtome-cryostat and transferred to slides. Sections were fixed with HistoChoice (Sigma-Aldrich, UK) for 20 min at room temperature and washed with PBS, before blocking with 5 % BSA/PBS for 60 min. Immunofluorescent co-staining of CXCR4 and cardiomyocytes was performed using rabbit monoclonal anti-CXCR4 (ab124824) and mouse anti-cardiac troponin T (ab8295) from Abcam (Gillingham, UK). Anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 555 secondary antibodies were purchased from Abcam. Cardiomyocyte co-stained samples were incubated in anti-CXCR4 and anti-cardiac troponin T, diluted in 1 % BSA/PBS 1:100 and 1:10 respectively, overnight at 4°C. Following washing and incubation with the appropriate secondary antibody diluted 1:400 in the same buffer for 60 min at room temperature, samples were washed again and coverslips mounted using fluorescence mounting medium (Dako, Ely, UK). 0.1 μg/ml Hoechst 33,258 nuclear stain (Life Technologies, Paisley, UK) was added with the secondary antibodies to all sections. Preparation of control sections was identical and they were incubated either with 1 % BSA/PBS only (unstained control) or with the relevant

secondary antibody in the absence of any primary antibody. After drying, Alexa 488 and Alexa 555 fluorescence was imaged using a 40× oil immersion objective, by sequential scanning using the 488 nm and 543 nm lines of a Leica SP5 confocal microscope and collecting emitted light at 500–530 nm and 580–650 nm respectively. Control experiments were performed to confirm the absence of fluorescence bleed-through or non-specific staining with secondary antibodies alone.

3.15 Statistical analysis

All data presented are presented as mean value ± SEM. Indistinguishable to previous researchers with the same model, a factorial one-way ANOVA was used for comparison between more than two groups in the functional recovery experiments. In cases where a significant F-value was obtained, Fisher's protected least significance difference (PLSD) post hoc was applied to test for significance. A p-value of <0.05 was taken to specify significance. With western blotting, protein band quantification are expressed as mean value ± SEM. The differences between groups have been tested

using a paired Student's t-test. All data were analysed using GraphPad Prism 6 (GraphPad Software Inc, California, USA).

Chapter 4: Characterisation - Establishing the human atrial trabecular superfusion model

4.1 Introduction

To test the hypothesis that SDF-1α may protect human myocardium from ischaemia-reperfusion injury, it was necessary to ensure that the trabecular model was a 'functioning' model. Previous investigators in the Hatter Cardiovascular Institute have employed the same model with various stabilisation techniques, hypoxia and re-oxygenation durations, in addition to different preconditioning protocols as tabulated below. It was therefore crucial to adopt a protocol that would function prior to studying the effects of SDF-1α was studied upon.

This chapter describes effort to define an optimum stabilisation time, a suitable preconditioning protocol and duration for the index hypoxia.

Author	Stabilisation (minutes)	Hypoxia (minutes)	Reoxygenation (minutes)	Preconditioning (minutes)
DM Walker	90	120	120	3+12
SD Morris	90	120	120	3+7
CS Carr	90	120	120	3+7
P Rees	90	120	120	3+7
ME S-Dick	90	120	120	3+7
V Sivaraman	75	120	120	4+16

Table 4.1. Protocol used by previous investigators at The Hatter Cardiovascular Institute. Preconditioning column consists of a period of hypoxia and re-oxygenation.

4.2 Hypothesis One

Human atrial trabeculae can be preconditioned using hypoxia and re-oxygenation.

4.3 Study subjects

There were eighty-three patients included in this set of experiments. All patients met the inclusion criteria as described earlier. The table below outlines the profile of patients that were included in this part of the study.

Profile		Value
Age		**60.6 years**
Gender	**Male**	**28%**
	Female	**72%**
Ethnicity	**White**	**73%**
	Asian	**27%**
Type of surgery	**Bypass only**	**55%**
	Valve only	**35%**
	Combined	**10%**
Previous MI		**8%**
Previous coronary intervention		**4%**

Table 4.2. Patient profile for hypothesis one.

4.4 Materials and methods

This is the same as described earlier. The only difference in this section was a period of preconditioning applied prior to subjecting the specimen to index hypoxia.

4.5 Objective one

To determine whether simulating ischaemia and re-oxygenation on the organ bath can precondition atrial trabeculae.

4.5.1 Experimental protocol

Muscle was stabilised as described earlier. Atrial trabeculae were suspended between a fixed glass hook and a force transducer then superfused in modified Tyrode's solution at 37°C whilst being field stimulated at 1 Hz. During the first part of stabilisation muscle a force of 0.1g was applied and muscle left for 30 minutes. This was followed by a stretch over 15 minutes to the peak of Frank-Starling curve followed by a final 45 minutes of stabilisation. **At this point a preconditioning protocol was applied**. The muscle was then subjected to a period of 90 minutes simulated hypoxia followed by

120 minutes re-oxygenation. This is depicted in the diagram below. Therefore trabeculae were randomly assigned to the following groups:

1. **Controls** (n≥5): Atrial trabeculae were subjected to 90 minutes of stabilisation, 90 minutes of simulated ischaemia (index hypoxia) and 120 minutes of simulated reperfusion (reoxygenation)

2. **Hypoxic preconditioning** (n≥8): Atrial trabeculae were subjected to the same protocol as the hypoxia-reoxygenation control in addition to a preconditioning protocol consisting of **3 minutes hypoxia, 12 minutes reoxygenation** prior to the index 90 minutes hypoxia.

3. **Sham** (n≥1): Atrial trabeculae were allowed to contract for a minimum of 2-hours following the stabilisation phase in the organ bath where they were superfused with oxygenated modified Tyrode's solution and paced at 1 Hz without any further experimental manipulation.

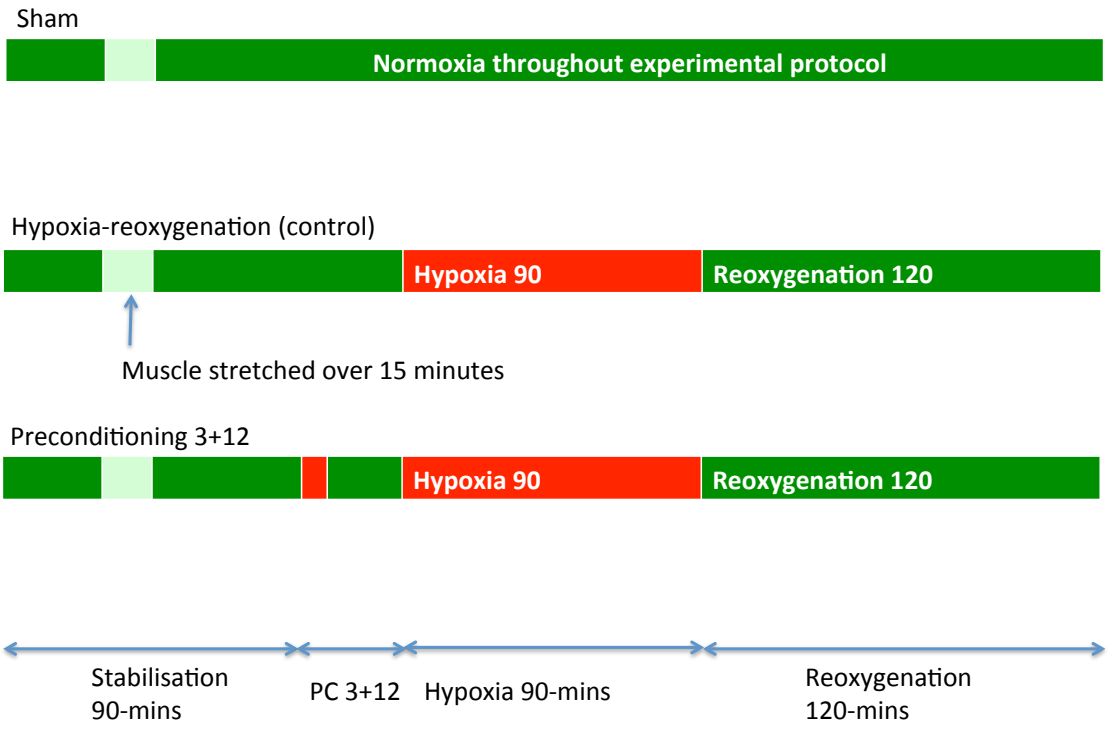


Figure 4.1. Experimental protocols for objective one.

4.5.2 Results

4.5.2.1 Patient profile

A total of 14 patients were included for this set of experiments including 10 males and 4 females, ages ranging from 51 to 76 years, inclusive. 43 atrial trabeculae were isolated from these appendages of which 14 were included in the study. 29 trabeculae were excluded according to the exclusion criteria mentioned in section 3.12.

4.5.2.2 Baseline functional data

Trabeculae in all groups were comparable at baseline in terms of average baseline force of contraction as shown in the table below. Baseline data was collected at the end of the 90 minutes stabilisation period.

Group	**Number**	**Average contraction in grams)**
Sham	**1**	**1.5**
Control	**5**	**0.86±0.09**
Precondition 3+12	**8**	**0.86±0.13**

Table 4.3. Baseline function data for preconditioning (3+12) experiments.

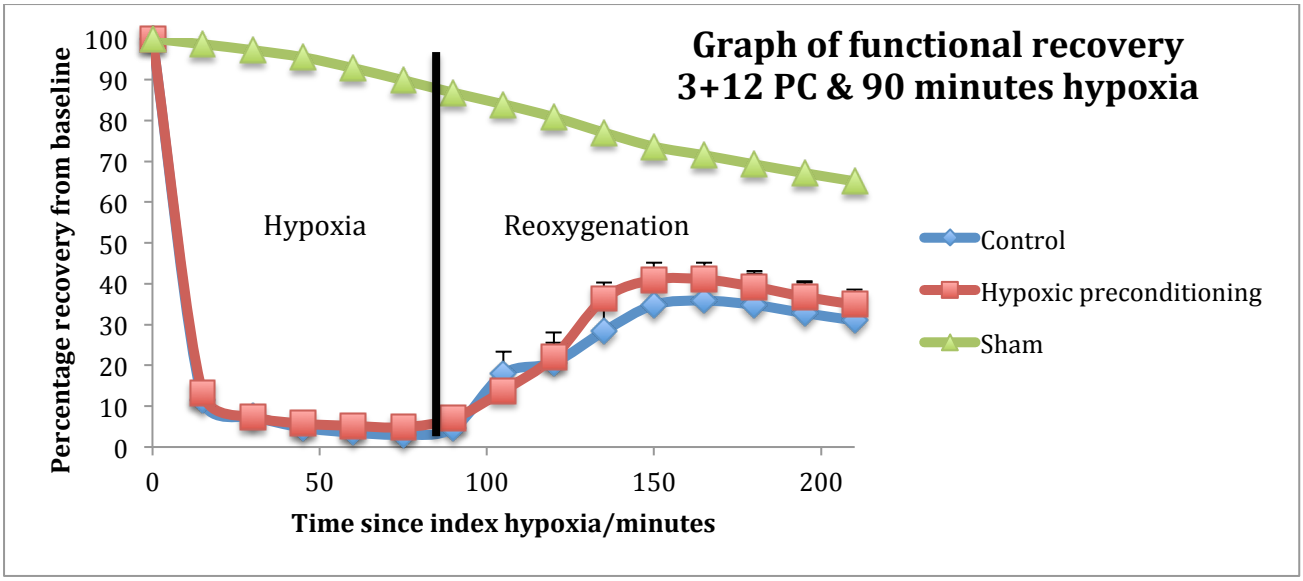


Figure 4.2. Graph showing recovery of function from onset of hypoxia.

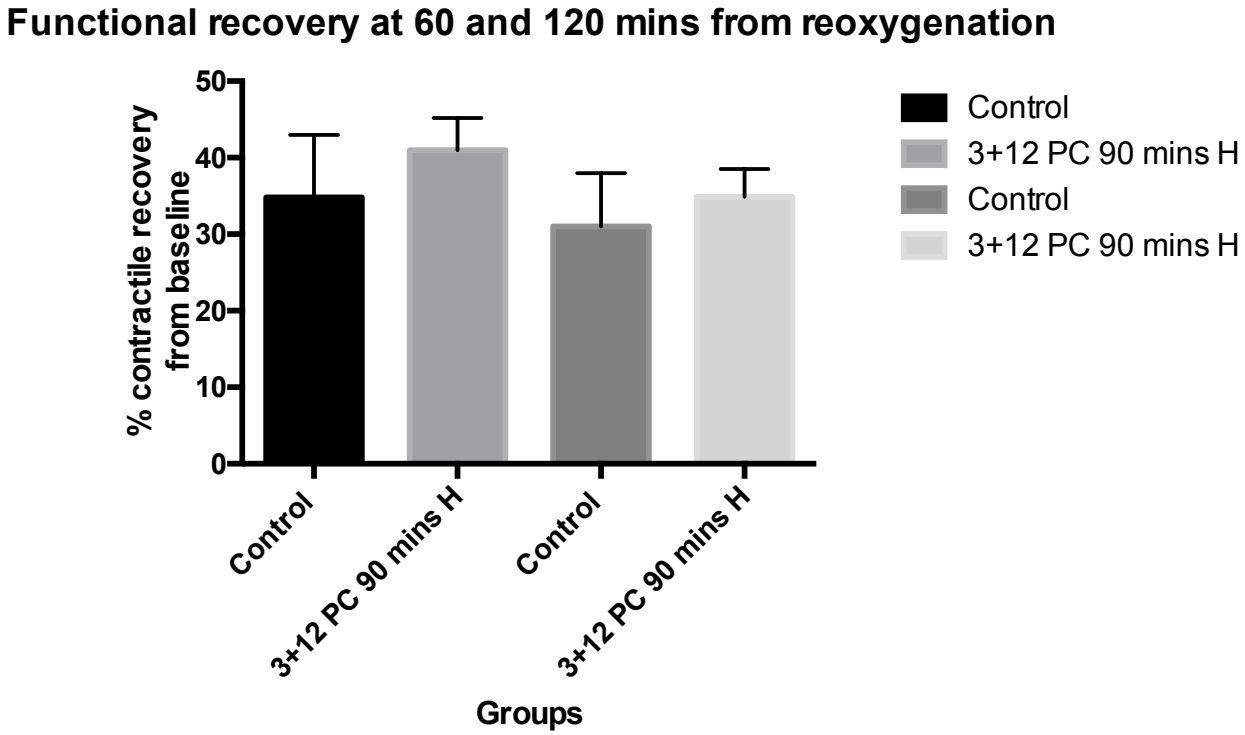


Figure 4.3. Bar chart representing recovery of function at 60 minutes and 120 minutes.

4.5.2.3 Recovery of function

Preconditioning with 3 minutes hypoxia and 12 minutes reoxygenation with index hypoxia duration of 90 minutes.

Preconditioning using the above protocol produced a recovery of function of 34.9 ± 3.6%. This was not significantly different to that of the control group, 31.0 ± 7.0%. There was no significant difference in recovery if data analysis was performed at 60 minutes or 120 minutes into re-oxygenation, control 34.8 ± 8.2% and 31 ± 7.0%, preconditioning 41.0 ± 4.2% and 34.9 ± 3.6%.

4.6 Objective Two

To determine whether increasing the preconditioning protocol achieves adequate protection of human muscle from the deleterious effects of hypoxia-reoxygenation injury.

4.6.1 Experimental protocol

The identical set-up was used as described in objective one except an enhanced preconditioning protocol was used as follows:

1. **Controls** (n≥5): Atrial trabeculae were subjected to 90 minutes of stabilisation, 90 minutes of simulated ischaemia (index hypoxia) and 120 minutes of simulated reperfusion (reoxygenation).

2. **Hypoxic preconditioning** (n≥4): Atrial trabeculae were subjected to the same protocol as the hypoxia-reoxygenation control in addition to a longer preconditioning protocol consisting of **4 minutes hypoxia, 10 minutes re-oxygenation** prior to the index 90 minutes hypoxia.

3. **Sham** (n≥1): Atrial trabeculae were allowed to contract for a minimum of 2-hours following the stabilisation phase in the organ bath where they were superfused with oxygenated.

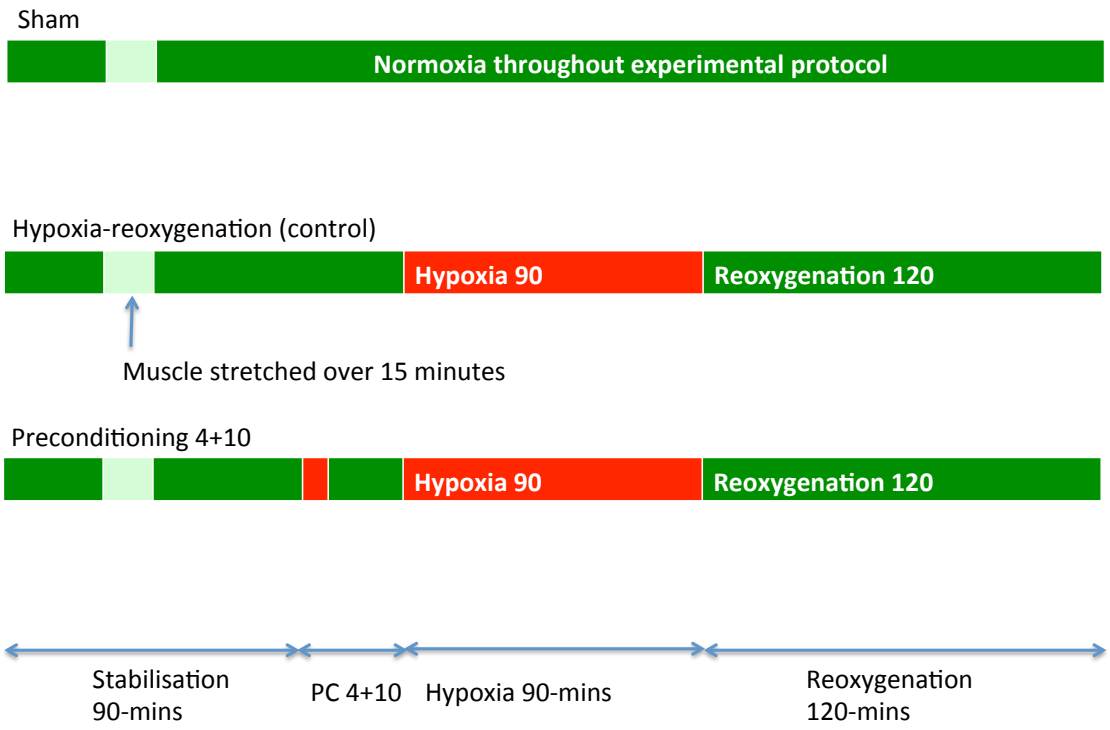


Figure 4.4. Experimental protocols for objective two

4.6.2 Results

4.6.2.1 Patient profile

A total of 10 patients were included for this set of experiments including 5 males and 5 females, ages ranging from 51 to 76 years,

inclusive. 33 atrial trabeculae were isolated from these appendages of which 10 were included in the study. 23 trabeculae were excluded according to the exclusion criteria mentioned in section 3.12.

4.6.2.2 Baseline functional data

Trabeculae in all groups were comparable at baseline in terms of average baseline force of contraction as shown in the table below. Baseline data was collected at the end of the 90 minutes stabilisation period.

Group	**Number**	**Average contraction in grams)**
Sham	**1**	**1.5**
Control	**5**	**0.86±0.09**
Precondition 4+10	**4**	**0.79±0.10**

Table 4.4. Baseline function data for preconditioning (4+10) experiments.

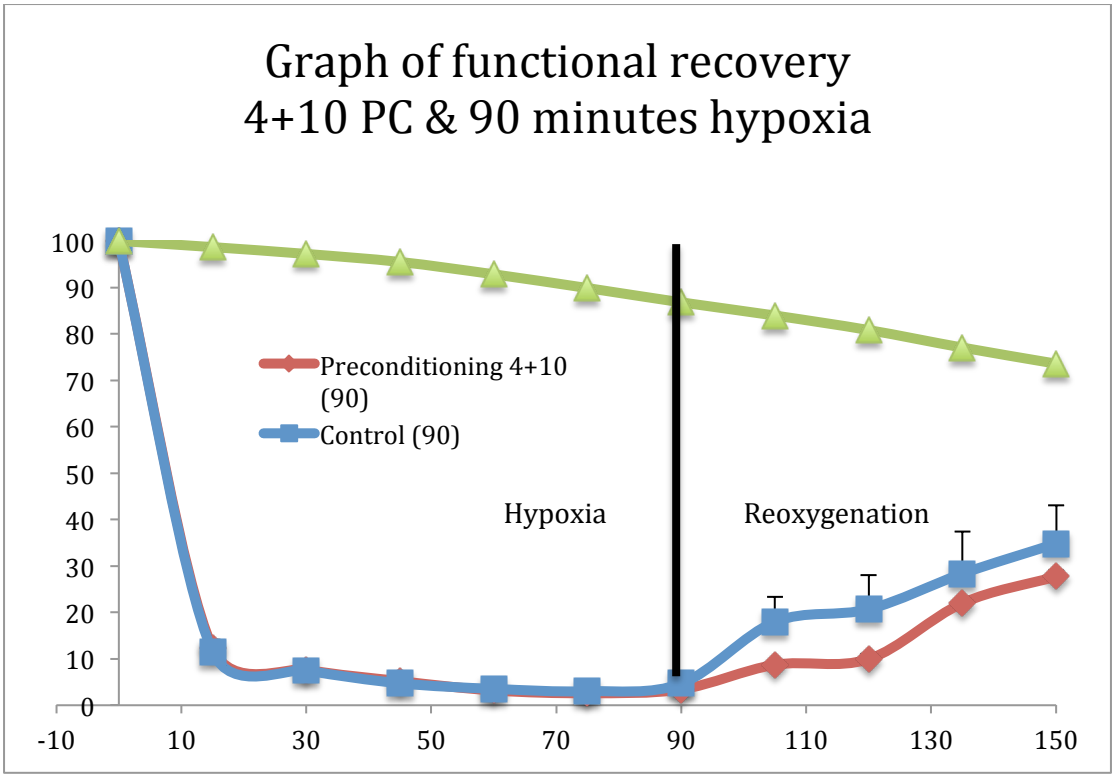


Figure 4.5. Graph showing recovery of function from onset of hypoxia.

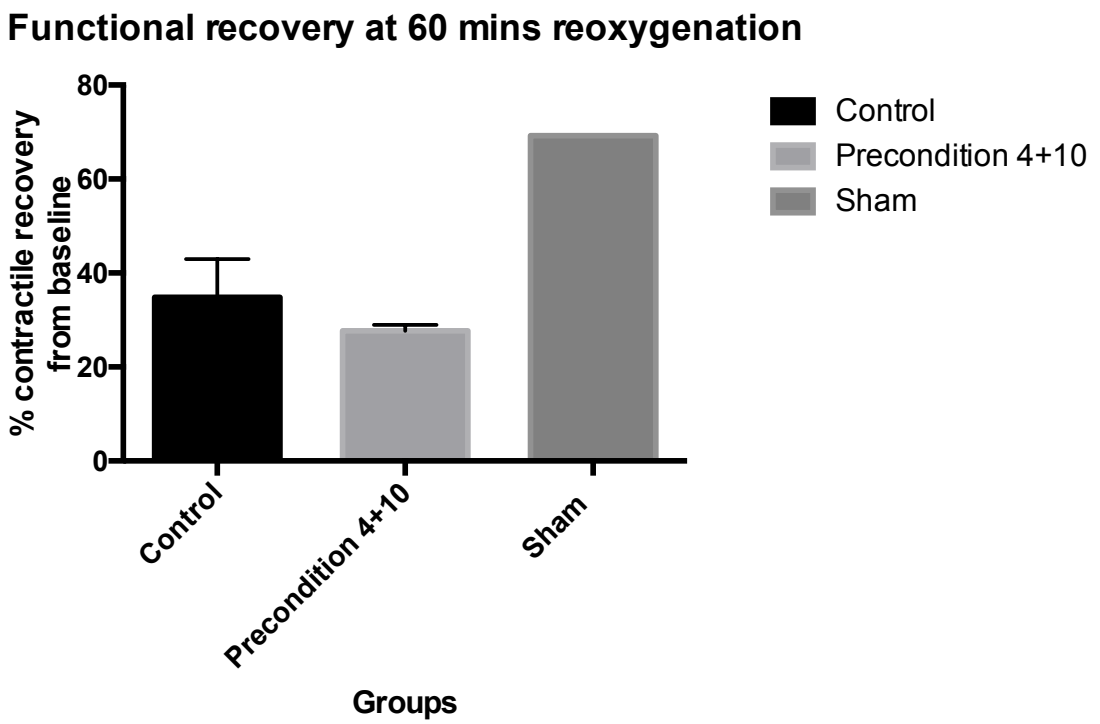


Figure 4.6. Bar chart representing recovery of function at 60 minutes since reoxygenation.

4.6.2.3 Recovery of function

Preconditioning with 4 minutes hypoxia and 10 minutes reoxygenation with index hypoxia duration of 90 minutes.

Preconditioning using the above protocol produced a recovery of function of 27.7 ± 1.2% at 60 minutes into reoxygenation. This was not significantly different to that of the control group, 34.8 ± 8.2%, $p>0.05$.

4.7 Objective Three

To determine whether further increasing the preconditioning protocol achieves adequate protection of human muscle from the deleterious effects of hypoxia-reoxygenation injury.

4.7.1 Experimental protocol

The identical set-up was used as described in objective two except an enhanced preconditioning protocol was used as follows:

1. **Controls** (n≥5): Atrial trabeculae were subjected to 90 minutes of stabilisation, 90 minutes of simulated ischaemia (index hypoxia) and 120 minutes of simulated reperfusion (reoxygenation).

2. **Hypoxic preconditioning** (n≥4): Atrial trabeculae were subjected to the same protocol as the hypoxia-reoxygenation control in addition to a longer preconditioning protocol consisting of **4 minutes hypoxia, 16 minutes re-oxygenation** prior to the index 90 minutes hypoxia.

3. **Sham** (n≥1): Atrial trabeculae were allowed to contract for a minimum of 2-hours following the stabilisation phase in the organ bath where they were superfused with oxygenated.

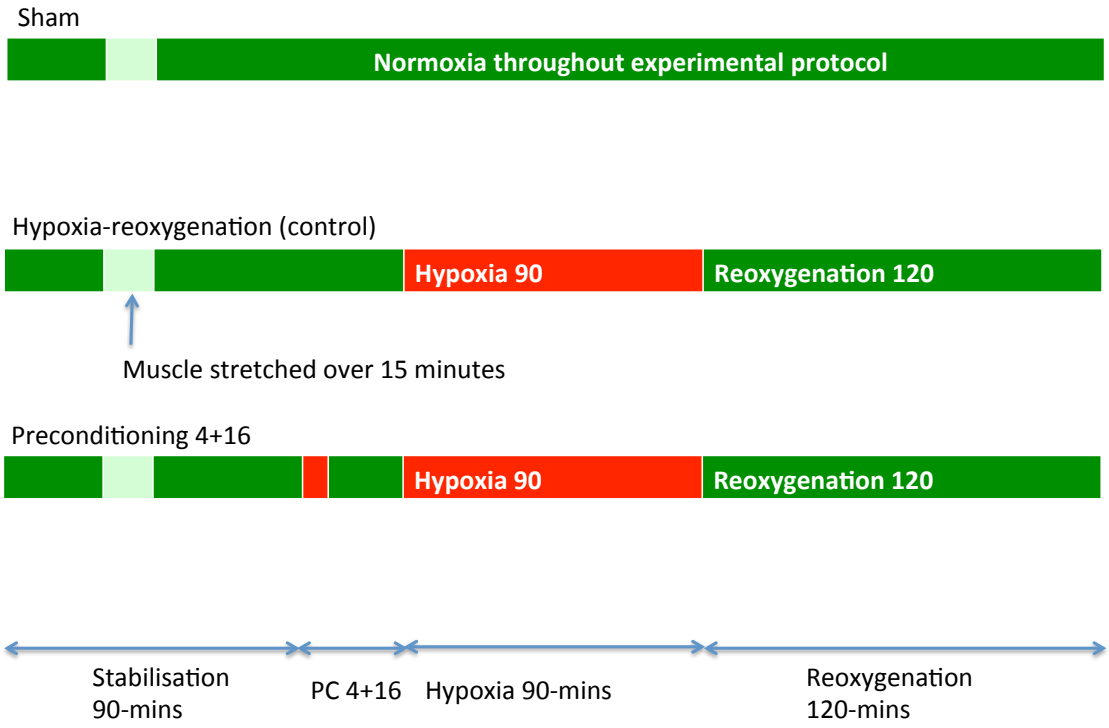


Figure 4.7. Experimental protocols for objective three.

4.7.2 Results

4.7.2.1 Patient profile

A total of 10 patients were included for this set of experiments including 9 males and 1 females, ages ranging from 51 to 76, inclusive. 33 atrial trabeculae were isolated from these appendages of which 10 were included in the study. 23 trabeculae were excluded according to the exclusion criteria mentioned in section 3.12.

4.7.2.2 Baseline functional data

Trabeculae in all groups were comparable at baseline in terms of average baseline force of contraction as shown in the table below. Baseline data was collected at the end of the 90 minutes stabilisation period.

Group	**Number**	**Average contraction in grams)**
Sham	**1**	**1.50**
Control	**5**	**0.86±0.19**
Precondition 4+16	**4**	**0.82±0.23**

Table 4.5. Baseline function data for preconditioning (4+16) experiments.

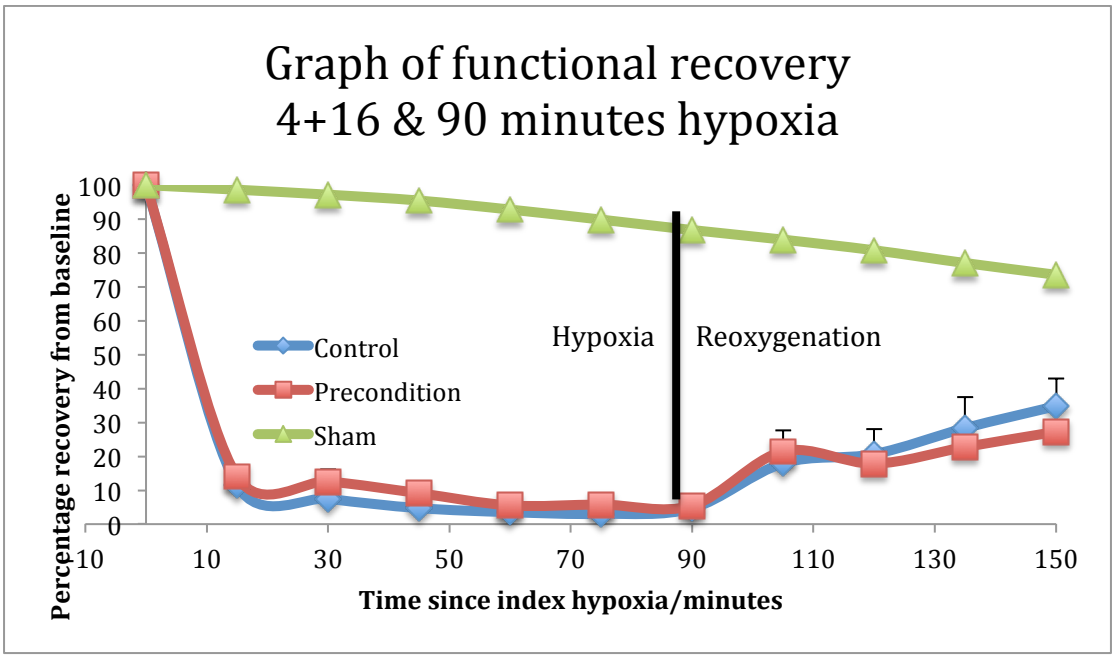


Figure 4.8. Graph showing recovery of function from onset of hypoxia.

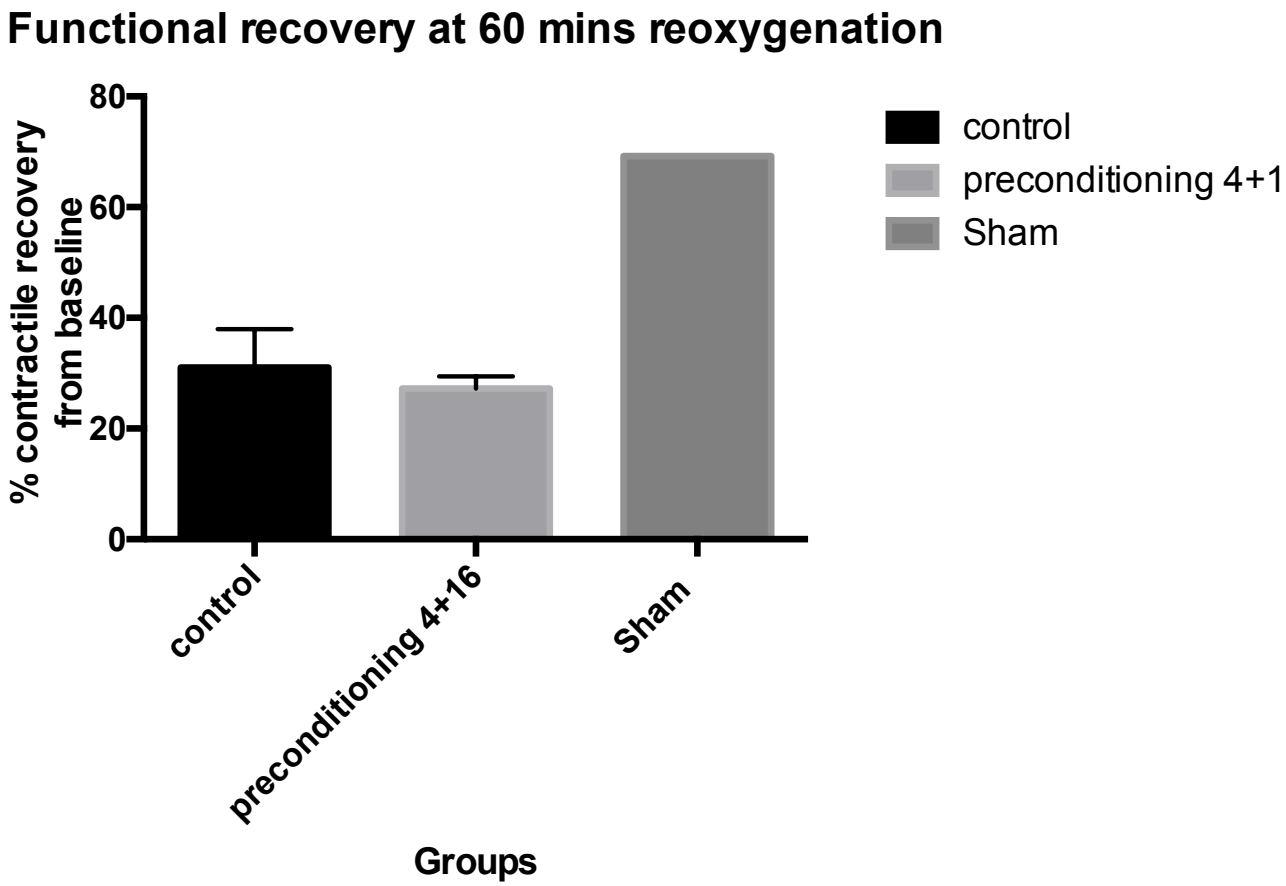


Figure 4.9. Bar chart representing recovery of function at 60 minutes since reoxygenation.

4.7.2.3 Recovery of function

Preconditioning with 4 minutes hypoxia and 16 minutes reoxygenation with index hypoxia duration of 90 minutes.

Preconditioning using the above protocol produced a recovery of function of 27.2 ± 2.2%. This was not significantly different to that of the control group, 34.8 ± 8.2%, $p>0.05$.

4.8 Objective Four

To determine the appropriate stabilisation time following isolation of trabeculae.

Previous investigators have mostly used 90 minutes stabilisation times prior to any form of intervention. The purpose of this section was to determine whether this period could be shortened to enable maximum time to conduct experiment on the trabeculae. Stability was determined by assessing whether there was a change in levels of Akt and Erk with time prior to evaluating the effect of SDF-1α on functional recovery.

4.8.1 Experimental protocol

Atrial trabeculae obtained during surgery were transported back to the laboratory as described under general methods. The sample was sectioned equally into five pieces and randomly assigned to one of five organ baths.

The protocol assigned to each bath were as follows:

1. Sample left in circulating buffer for 10 minutes.

2. Sample left in circulating buffer for 30 minutes.

3. Sample left in circulating buffer for 60 minutes.

4. Sample left in circulating buffer for 60 minutes with 1μM insulin (1nM in cell studies).

5. Sample left in circulating buffer for 120 minutes.

Following the ascribed period in the organ bath the specimen would be snap frozen in liquid nitrogen then stored in -80°C freezer until protein was extracted.

4.8.2 Results

4.8.2.1 Patient profile

A total of 4 patients were included for this set of experiments including 4 males and 0 females, ages ranging from 46 to 78 years, inclusive. There were equal numbers of patients undergoing either CABG or aortic valve surgery. The mean weight of the atrial

appendage obtained for each experiment was 1.08±0.20g and the mean weight of specimen placed in individual organ bath was 0.34±0.02g. All patients had a medical history consisting of hypertension and hyperlipidaemia.

4.8.2.2 Western blots

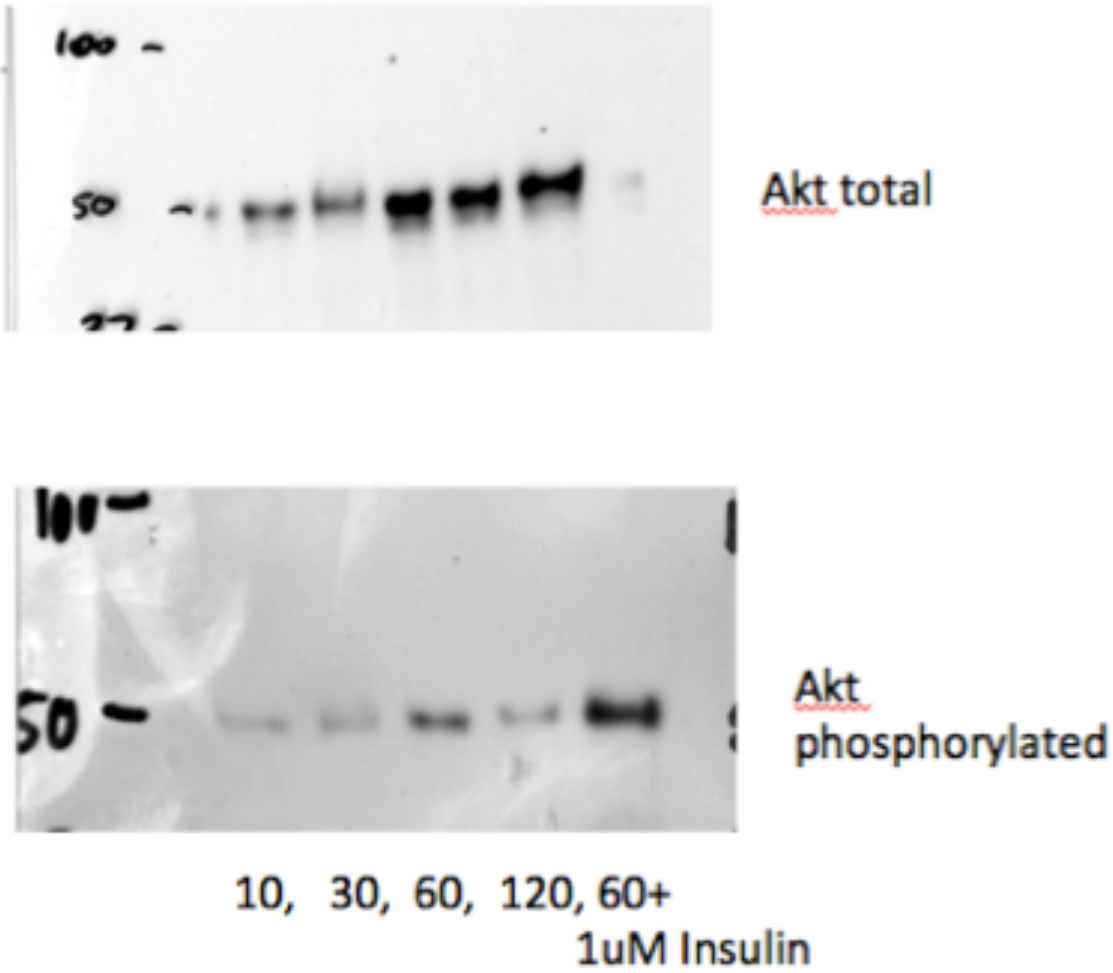


Figure 4.10. Western blots for Akt and phosphorylated Akt

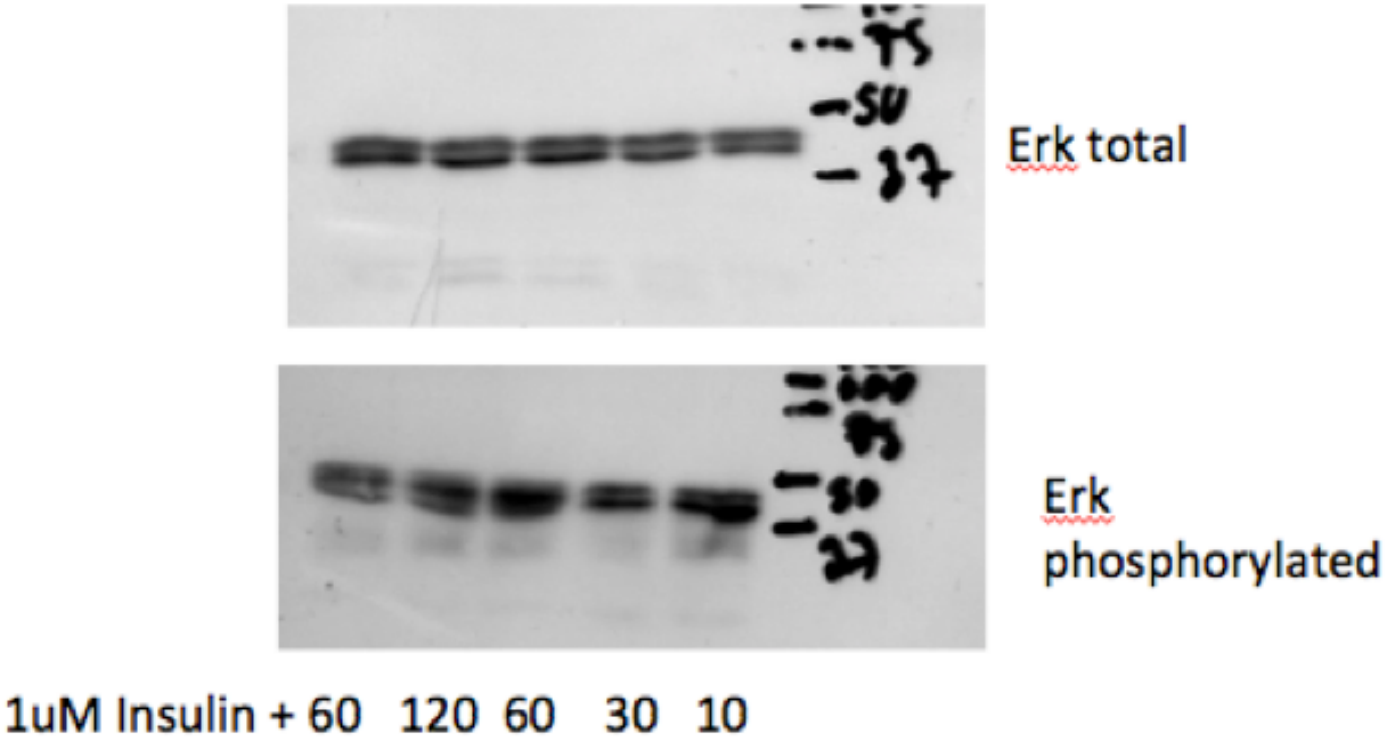


Figure 4.11. Western blots for Erk and phosphorylated Erk

4.8.2.3 Densitometry

	AKT Phospo	AKT Total	Akt P/T	ERK Phospho	ERK Total	Erk P/T
Patient 1						
10	17089.57	18889.41	0.904717	27448.15	16037.24	1.711526
30	5209.317	9208.966	0.565679	17218.92	17335.31	0.993286
60	3129.468	19705.12	0.158815	21819.03	20105.97	1.085202
120	2429.69	29233.36	0.083114	17279.79	20561.33	0.840402
60 + Insulin	7855.752	23461.78	0.334832	13028.02	19318.02	0.674397
Patient 2						
10	17132.11	18125.69	0.945184	33663.24	24242.82	1.388586
30	17528.23	32281.91	0.542974	21095.47	21003.28	1.004389
60	4821.731	6862.731	0.702597	14349.94	14201.89	1.010424
120	4116.075	9067.43	0.453941	18950.37	13867.21	1.36656
60 + Insulin	23481.71	23823.58	0.98565	16861.84	11248.05	1.49909
Patient 3						
10	1465.134	5697.731	0.257143	18259.08	10941.65	1.668768
30	1946.79	4586.731	0.42444	26222.58	25693.69	1.020584
60	6518.974	21723.79	0.300085	16422.42	21296.69	0.771125
120	3138.418	18644.49	0.168329	18423.52	13792.79	1.335735
60 + Insulin	17478.28	22954.76	0.761423	28985.47	13463.31	2.152923
Patient 4						
10	10645.95	34928	0.304797	10922.02	20776.02	0.525703
30	9307.681	12176.99	0.764367	21728.47	16313.6	1.331924
60	10726	23328.74	0.459776	13173.02	20374.69	0.646538
120	3846.368	9421.258	0.408265	15006.57	22352.52	0.671359
60 + Insulin	21882.32	28187.69	0.776308	17624.21	21920.72	0.803998

Figure 4.12. Densitometry analysis using Image J.

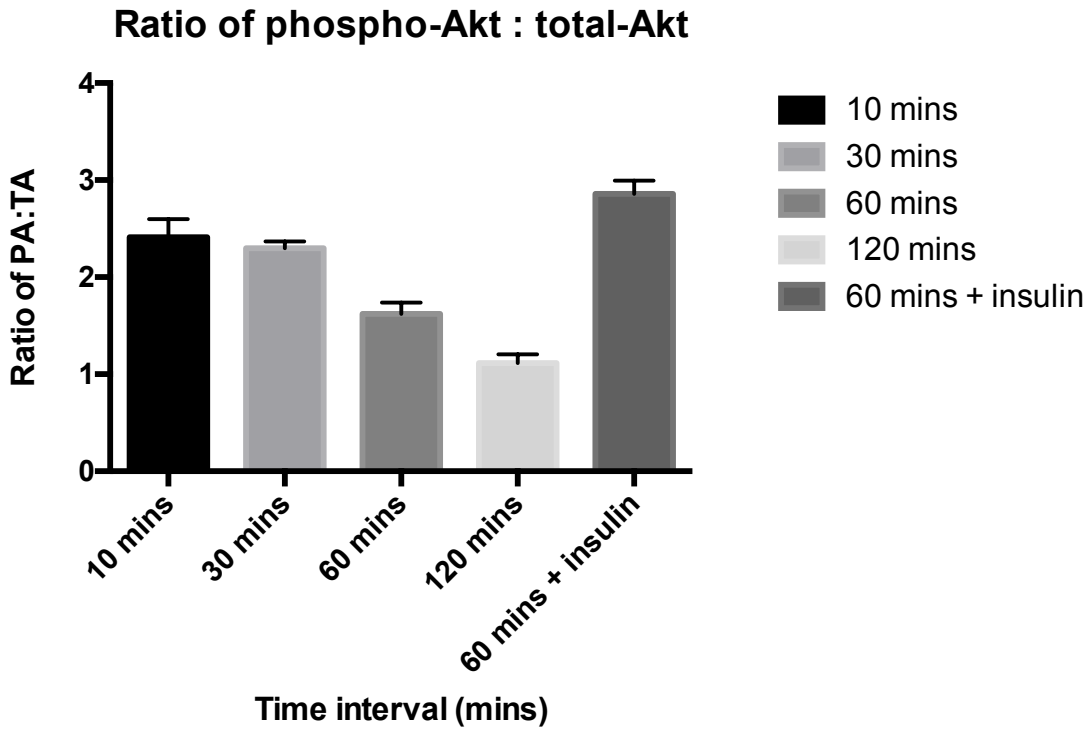


Figure 4.13. Ratio of phosphorylated Akt to total Akt.

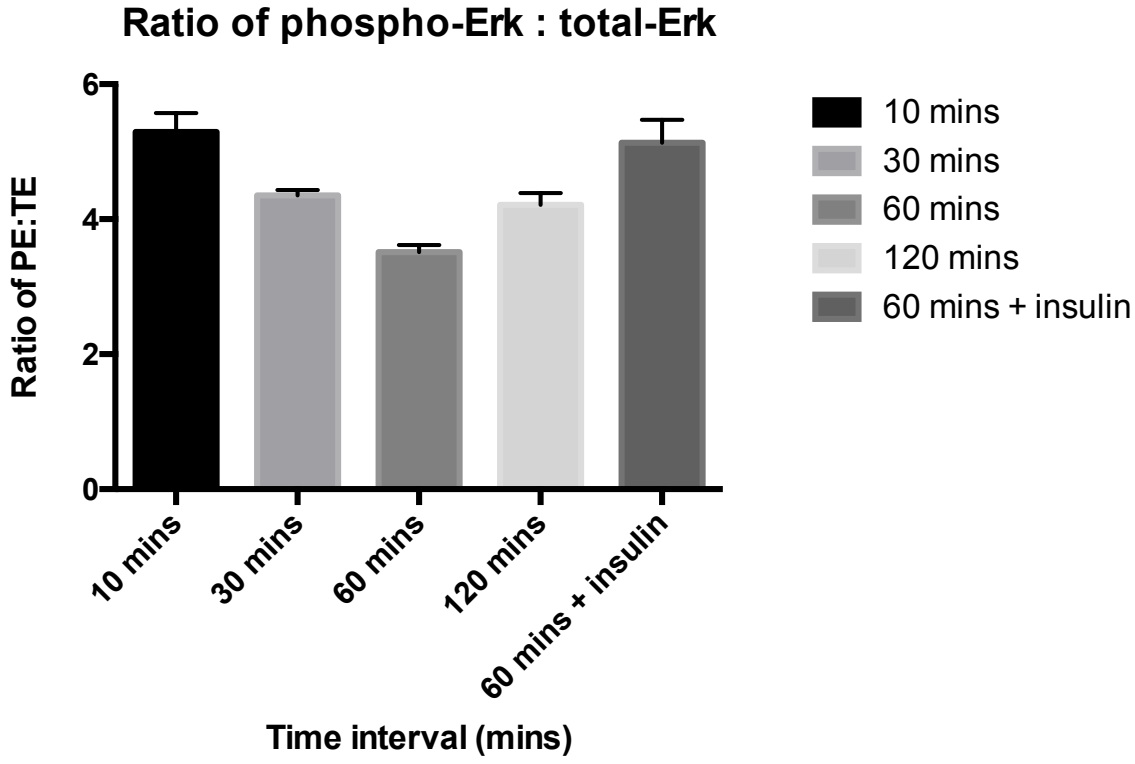


Figure 4.14. Ratio of phosphorylated Erk to total Erk.

4.8.2.4 Ratio of phosphorylated kinase to total kinase present

The figures 28 and 29 demonstrate the ratio phosphorylated Akt or Erk to total Akt or Erk respectively. Ratios were higher at 10 and 30 minutes compared to 60 minutes. There was no significant difference between ratios at 60 minutes to 120 minutes. Insulin was used as a positive control.

4.9 Objective Five

To determine whether reducing the index hypoxia duration affects the ability to precondition human muscle.

All experiments conducted with the 90 minutes hypoxia duration have failed to demonstrate the ability for hypoxic preconditioning to protect human tissue. The question was whether the 90 minutes of hypoxia injured the muscle to such an extent that prevents it being successfully preconditioned. Therefore protocol that consisted of shorter durations of index hypoxia was examined in this section. The objective was to cause enough hypoxic injury to allow muscle to be preconditioned successfully.

4.9.1 Experimental protocol

The same standard stabilisation technique was used here as with other experiments. Different index hypoxia durations were employed upon here. All muscles were then reoxygenated for 2 hours. Trabeculae were randomly assigned to one of 2 protocols (see figure 25):

1. **Control with 45 minutes index hypoxia** (n≥4): Atrial trabeculae were subjected to 90 minutes of stabilisation, **45 minutes of simulated ischaemia** (index hypoxia) and 120 minutes of simulated reperfusion (reoxygenation)

2. **Control with 60 minutes index hypoxia** (n≥5): Atrial trabeculae were subjected to 90 minutes of stabilisation, **60 minutes of simulated ischaemia** (index hypoxia) and 120 minutes of simulated reperfusion (reoxygenation).

3. **Sham** (n≥1): Atrial trabeculae were allowed to contract for a minimum of 2-hours following the stabilisation phase in the organ bath where they were superfused with oxygenated modified Tyrode's solution and paced at 1 Hz without any further experimental manipulation. Muscle was stretched to the peak of the Frank-Starling curve.

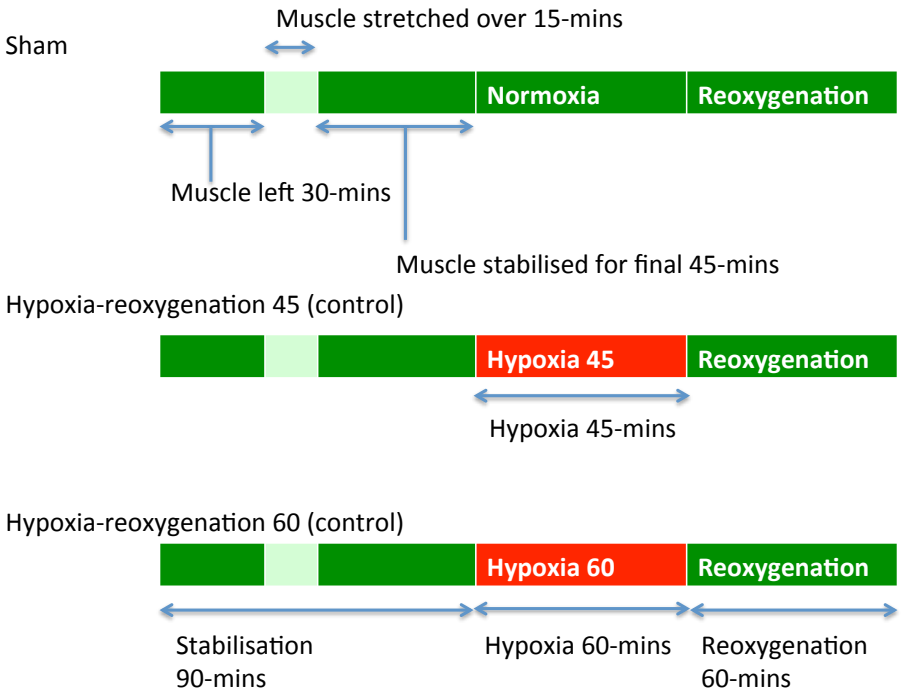


Figure 4.15. Experimental protocol for objective five.

4.9.2 Results

4.9.2.1 Patient profile

A total of 10 patients were included for this set of experiments including 10 males and 0 females, ages ranging from 50 to 79, inclusive. 18 atrial trabeculae were isolated from these appendages of which 10 were included in the study. Eight trabeculae were excluded according to the exclusion criteria mentioned in section 3.12.

4.9.2.2 Baseline functional data

Trabeculae in all groups were comparable at baseline in terms of average baseline force of contraction as shown in the table below. Baseline data was collected at the end of the 90 minutes stabilisation period.

Group	**Number**	**Average contraction in grams)**
Sham	**1**	**1.5**
Control 45	**4**	**0.75±0.10**
Control 60	**5**	**0.73±0.10**

Table 4.6. Baseline function data for varying hypoxia experiments.

4.9.2.3 Recovery of function

Index hypoxia duration

Index hypoxia duration of 45 and 60 minutes using the above protocol produced a significant difference in the recovery of function, 64.3 ± 2.5% and 32.0 ± 6.6% respectively, $p<0.05$.

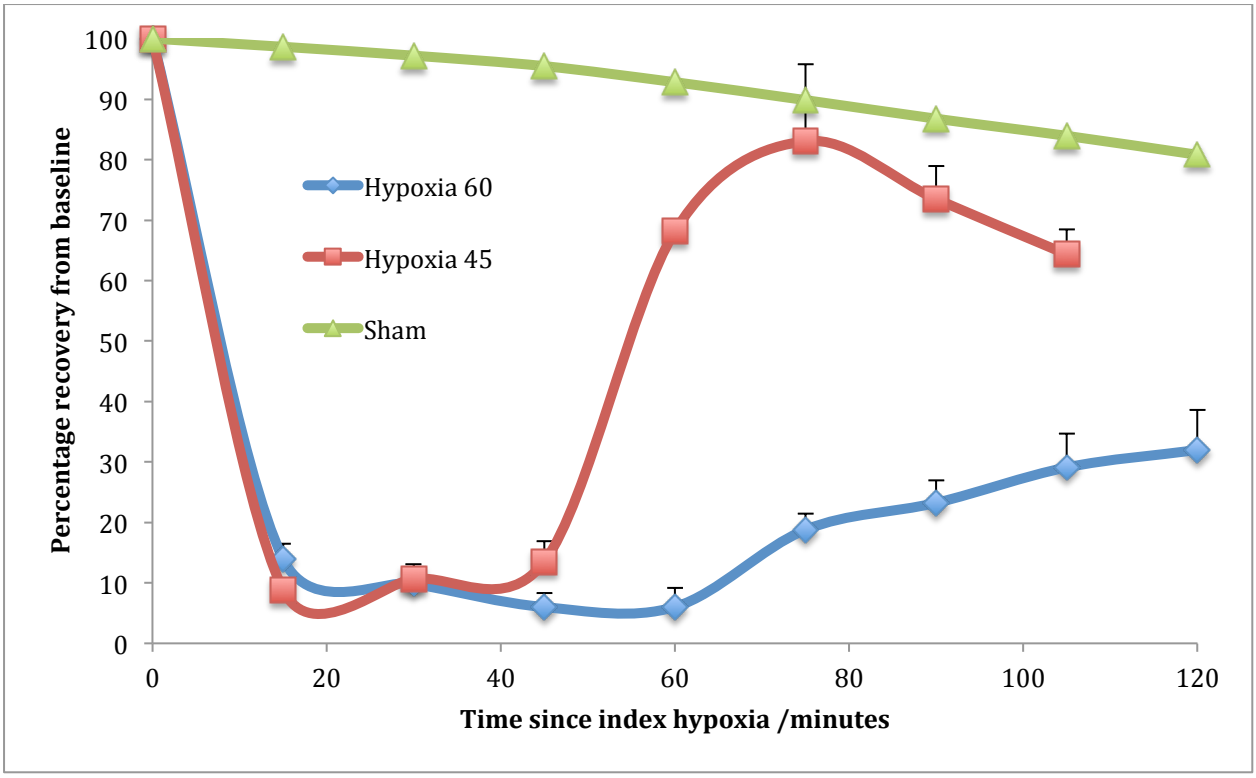


Figure 4.16. Graph comparing recovery of function when muscles are subjected to 45 or 60 minutes hypoxia.

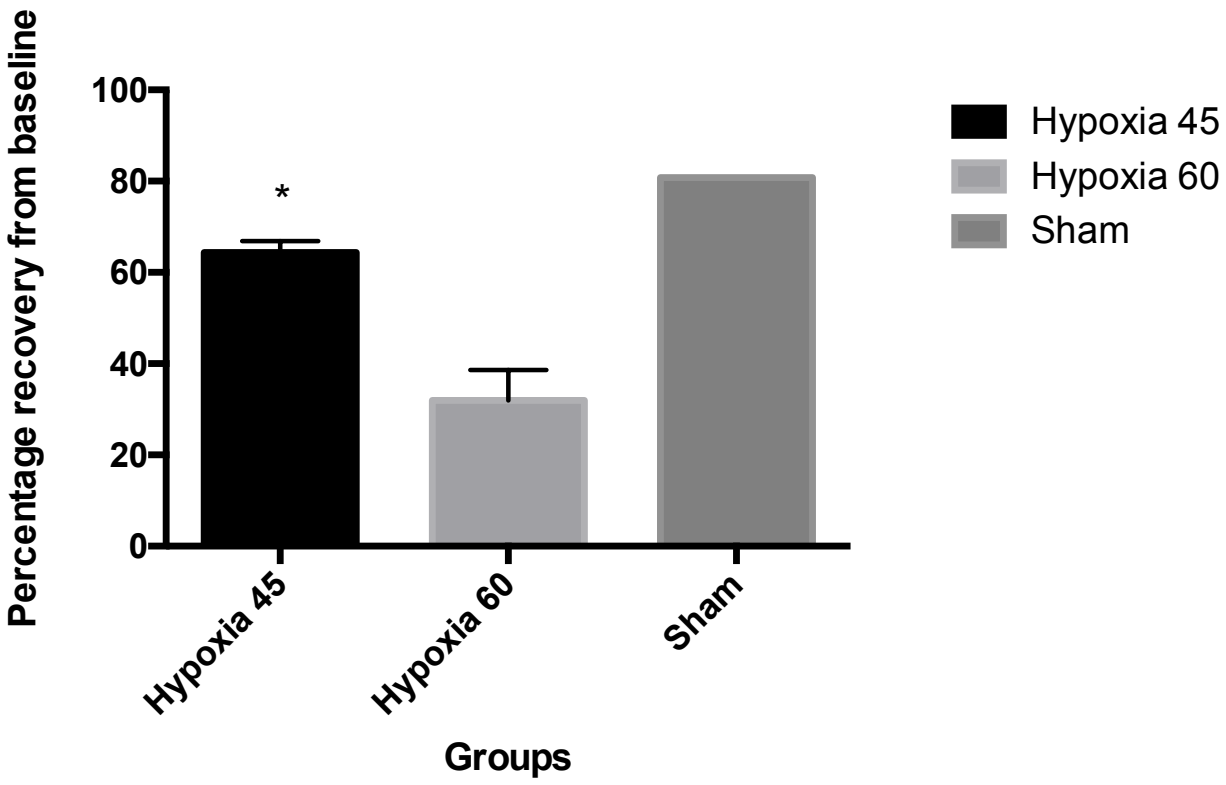


Figure 4.17. Bar chart comparing recovery of function when muscles are subjected to 45 or 60 minutes hypoxia.

4.10 Objective Six

To determine whether stretch during stabilisation affects the ability of human atrial muscle to be preconditioned.

4.10.1 Experimental protocol

The stabilisation technique varied during this set of experiments. In previous experimental protocols, the suspended atrial trabeculae would undergo stabilisation by applying 0.1g stretch at the beginning of the protocol to stimulate muscle contraction then being left for 30 minutes. The muscle would then be stretched over 15 minutes to the peak of Frank-Starling curve before allowing a final stabilisation phase of 45 minutes. It was following this stabilisation phase when experimental intervention would be applied.

In this experimental set the muscle was stretched immediately to 0.5g and a second stretch applied into 20 minutes of the protocol as the resting tension of the muscle would declining with a corresponding reduction in the developed force. The muscle would then be left for the remainder of the stabilisation phase. This is depicted in the diagram below. Trabeculae were randomly assigned to the following groups:

1. **Limited stretch** (n≥6): Atrial trabeculae were subjected to 90 minutes of stabilisation during which 0.5g stretch was applied at the start of the experiment followed by a second stretch at 20 minutes into the protocol. This was followed by 60 minutes of simulated ischaemia (index hypoxia) and 60 minutes of simulated reperfusion (reoxygenation).

2. **Hypoxic preconditioning** (n≥6): Atrial trabeculae that were stretched by a limited number of times were subjected to a preconditioning protocol consisting of **4 minutes hypoxia and 16 minutes reoxygenation** prior to the index hypoxia of 60 minutes.

3. **Sham** (n≥1): Atrial trabeculae were allowed to contract for a minimum of 2-hours following the stabilisation phase in the organ bath where they were superfused with oxygenated modified Tyrode's solution and paced at 1 Hz without any further experimental manipulation. A limited number of stretches (2) were applied within the first 45 minutes.

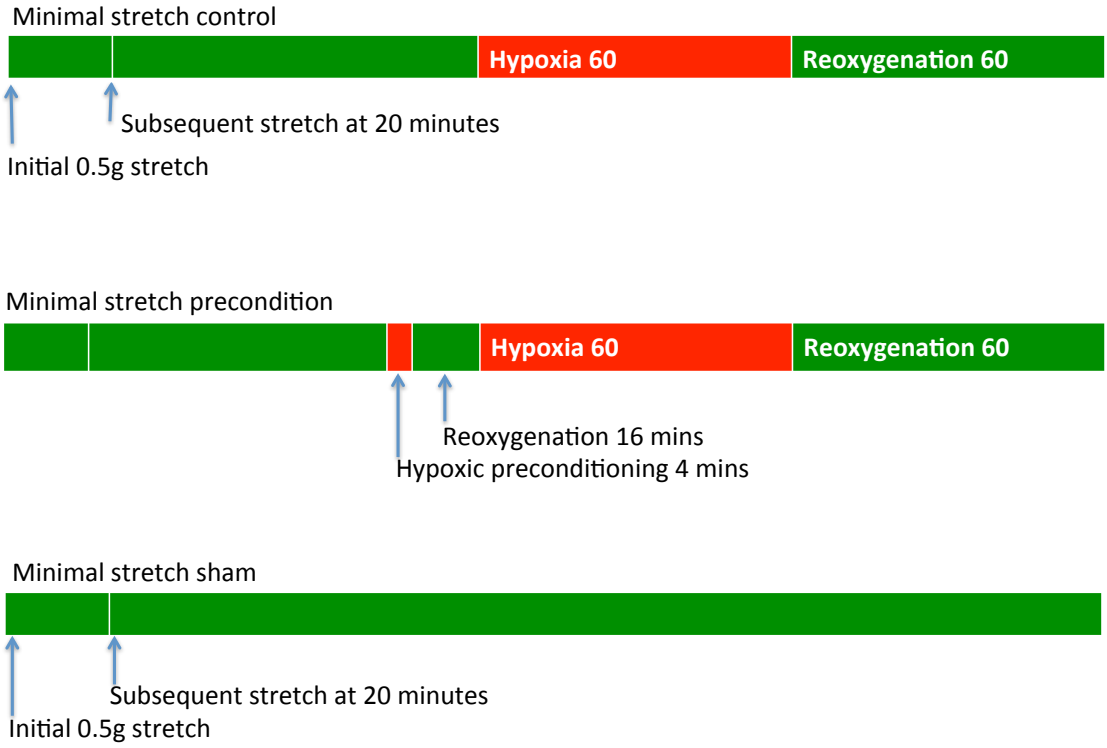


Figure 4.18. Experimental protocol for objective six.

4.10.2 Results

4.10.2.1 Patient profile

A total of 13 patients were included for this set of experiments including 12 males and 1 females, ages ranging from 49 to 80, inclusive. 40 atrial trabeculae were isolated from these appendages of which 13 were included in the study. 27 trabeculae were excluded according to the exclusion criteria mentioned in section 3.12.

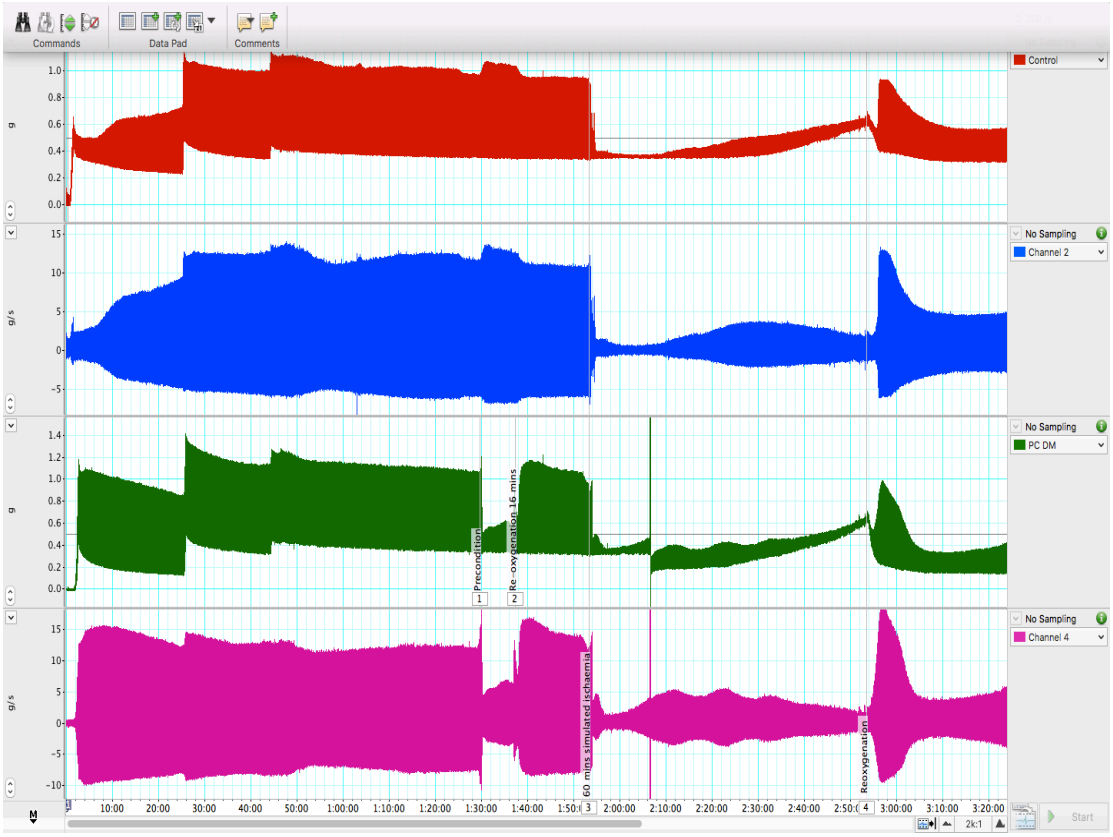


Figure 4.19. Screenshot of computer recording minimal stretch control (red) and preconditioning (green).

4.10.2.2 Baseline functional data

Trabeculae in all groups were comparable at baseline in terms of average baseline force of contraction as shown in the table below. Baseline data was collected at the end of the 90 minutes stabilisation period.

Group	**Number**	**Average contraction in grams)**
Sham	**1**	**1.5**
Control	**6**	**0.94 ± 0.11**
Precondition 4+16	**6**	**0.78±0.10**

Table 4.7. Baseline function data for preconditioning (4+16) experiments with minimal stretch.

4.10.2.3 Recovery of function

The effect of stretch on the ability to precondition human atrial trabeculae.

Preconditioning using the above protocol produced a recovery of function of 33.9 ± 3.9%. This was not significantly different to that of the control group, 31.1 ± 2.4%, $p>0.05$.

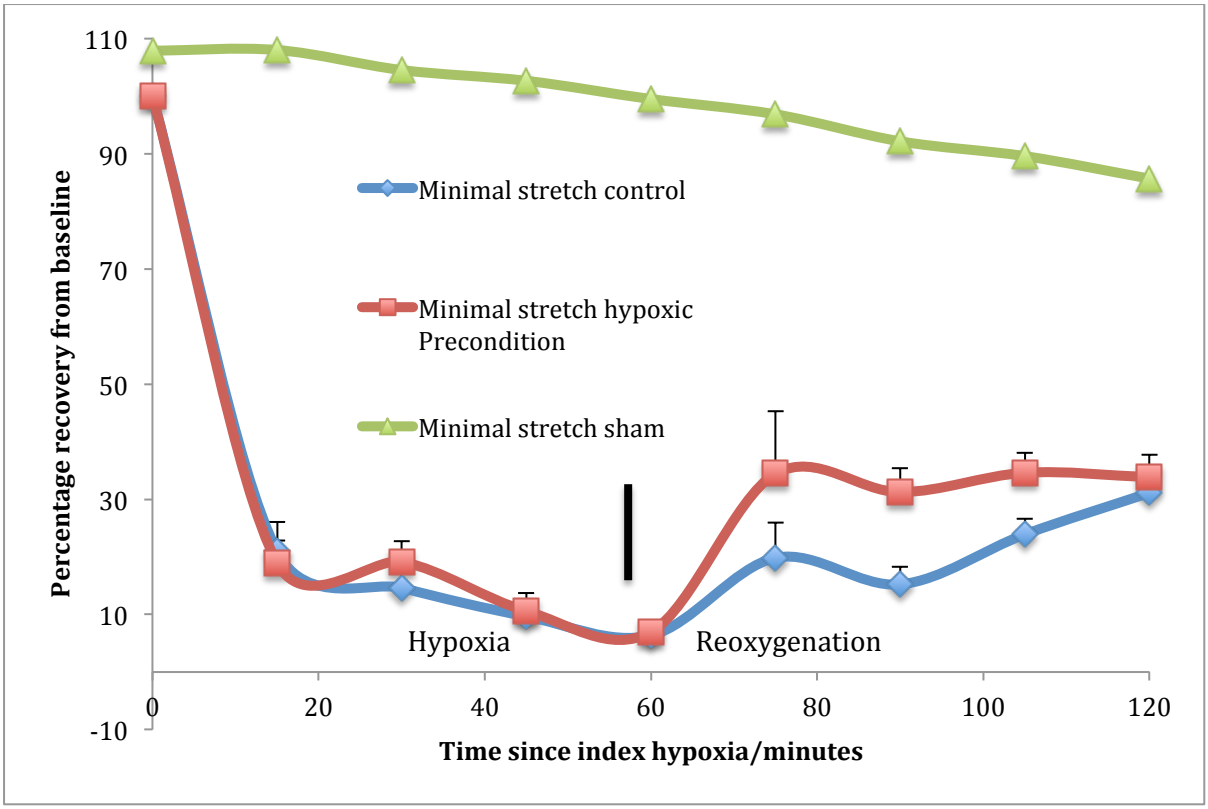


Figure 4.20. Graph showing recovery of function from onset of hypoxia for minimal stretch experiments.

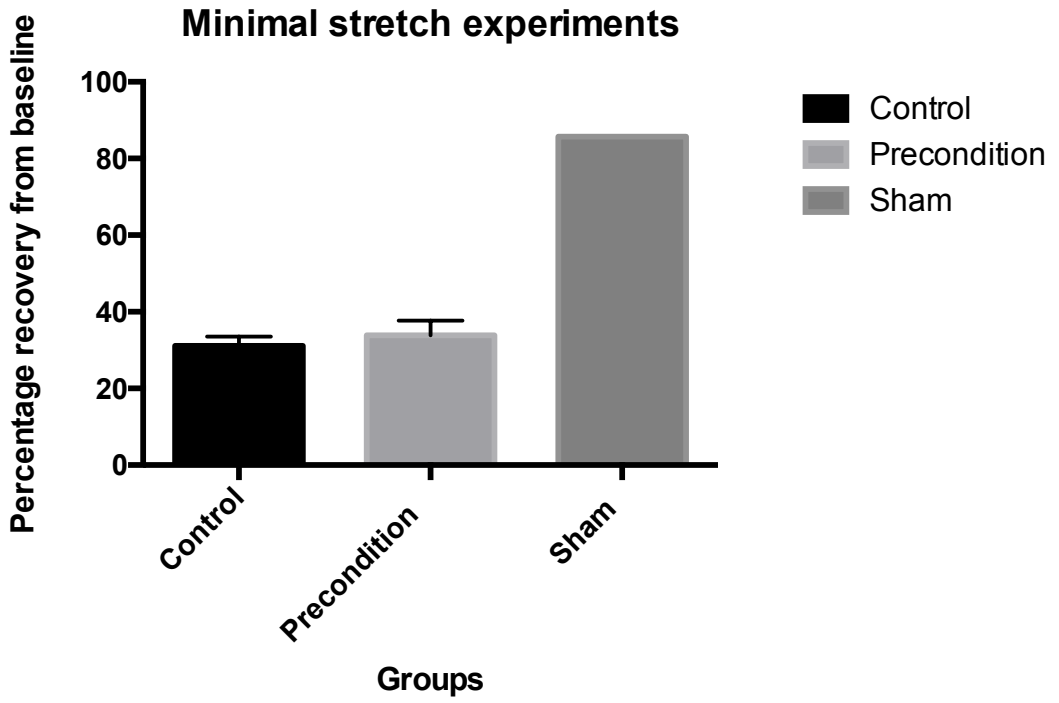


Figure 4.21. Bar chart representing recovery of function from baseline at 60 minutes for minimal stretch experiments.

4.11 Objective Seven

To determine whether human muscle can be preconditioned under optimised stabilisation and hypoxia times established earlier.

This final section of the human atrial characterisation explores whether atrial trabeculae can now be preconditioned having determined what is assumed to be an optimised stabilisation phase and a hypoxia time that is sufficient to cause muscle injury yet leave it with the capacity to undergo preconditioning and have enhanced recovery when compared to control specimens.

4.11.1 Experimental protocol

The same standard stabilisation technique was used here as with other experiments. The index hypoxia duration was 60 minutes. All muscles were then reoxygenated for 2 hours. Trabeculae were randomly assigned to one of 2 protocols (see figure 30):

1. **Control** (n≥11): Atrial trabeculae were subjected to 90 minutes of stabilisation, 60 minutes of simulated ischaemia (index

hypoxia) and 120 minutes of simulated reperfusion (reoxygenation)

2. **Hypoxic preconditioning** (n≥10): Atrial trabeculae were subjected to a preconditioning protocol consisting of **6 minutes hypoxia and 4.5 minutes reoxygenation** prior to the index hypoxia of 60 minutes.

3. **Sham** (n≥1): Atrial trabeculae were allowed to contract for a minimum of 2-hours following the stabilisation phase in the organ bath where they were superfused with oxygenated modified Tyrode's solution and paced at 1 Hz without any further experimental manipulation. Muscle was stretched to the peak of the Frank-Starling curve.

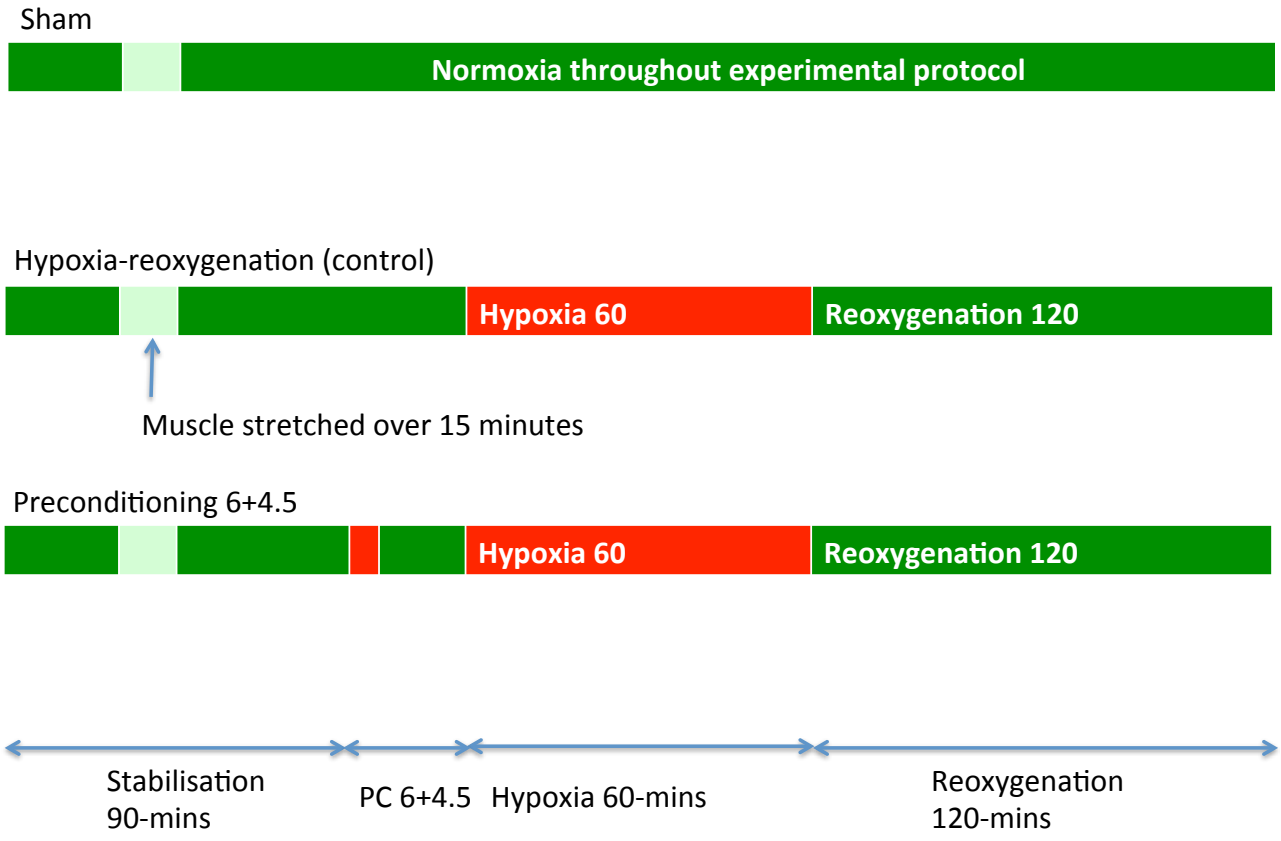


Figure 4.22. Experimental protocol for objective six.

4.11.2 Results

4.11.2.1 Patient profile

A total of 22 patients were included for this set of experiments including 22 males and 0 females, ages ranging from 45 to 79, inclusive. 43 atrial trabeculae were isolated from these appendages of which 22 were included in the study. Twenty-one trabeculae were excluded according to the exclusion criteria mentioned in section 3.12.

4.11.2.2 Baseline functional data

Trabeculae in all groups were comparable at baseline in terms of average baseline force of contraction as shown in the table below. Baseline data was collected at the end of the 90 minutes stabilisation period.

Group	Number	Average contraction (grams)
Sham	**1**	**1.5**
Control	**11**	**0.75±0.06**
Precondition	**10**	**0.74±0.07**

Table 4.8. Baseline function data for trabeculae subjected to preconditioning.

4.11.2.3 Recovery of function

Preconditioning using protocol 4+6.5 minutes

Preconditioning using the above protocol resulted in a significant improvement in the recovery of function compared to the control experiments, 47.6 ± 3.7 % and 26.9 ± 1.8 % respectively, $p<0.05$.

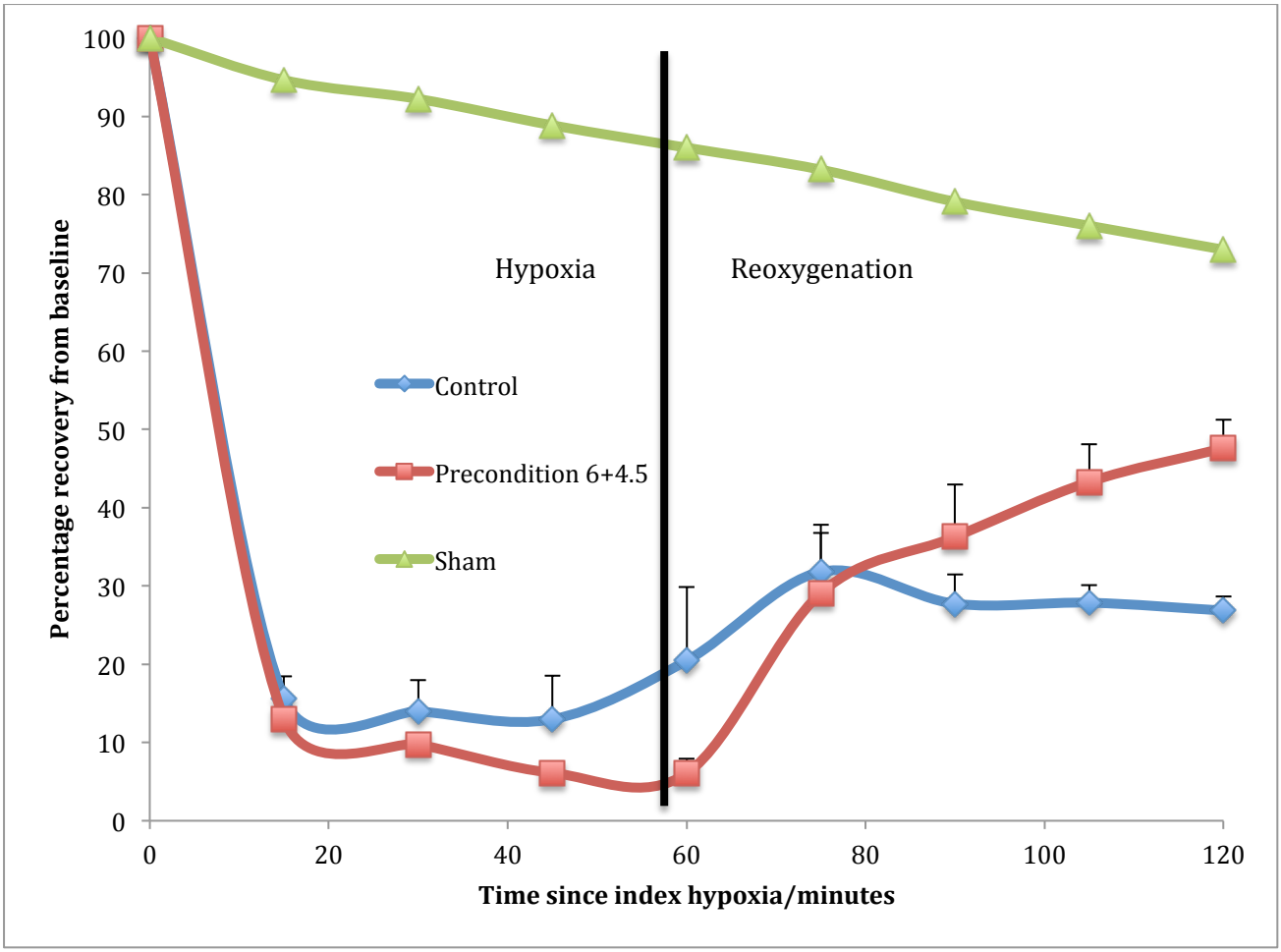


Figure 4.23. Recovery of function, control versus preconditioning 6+4.5

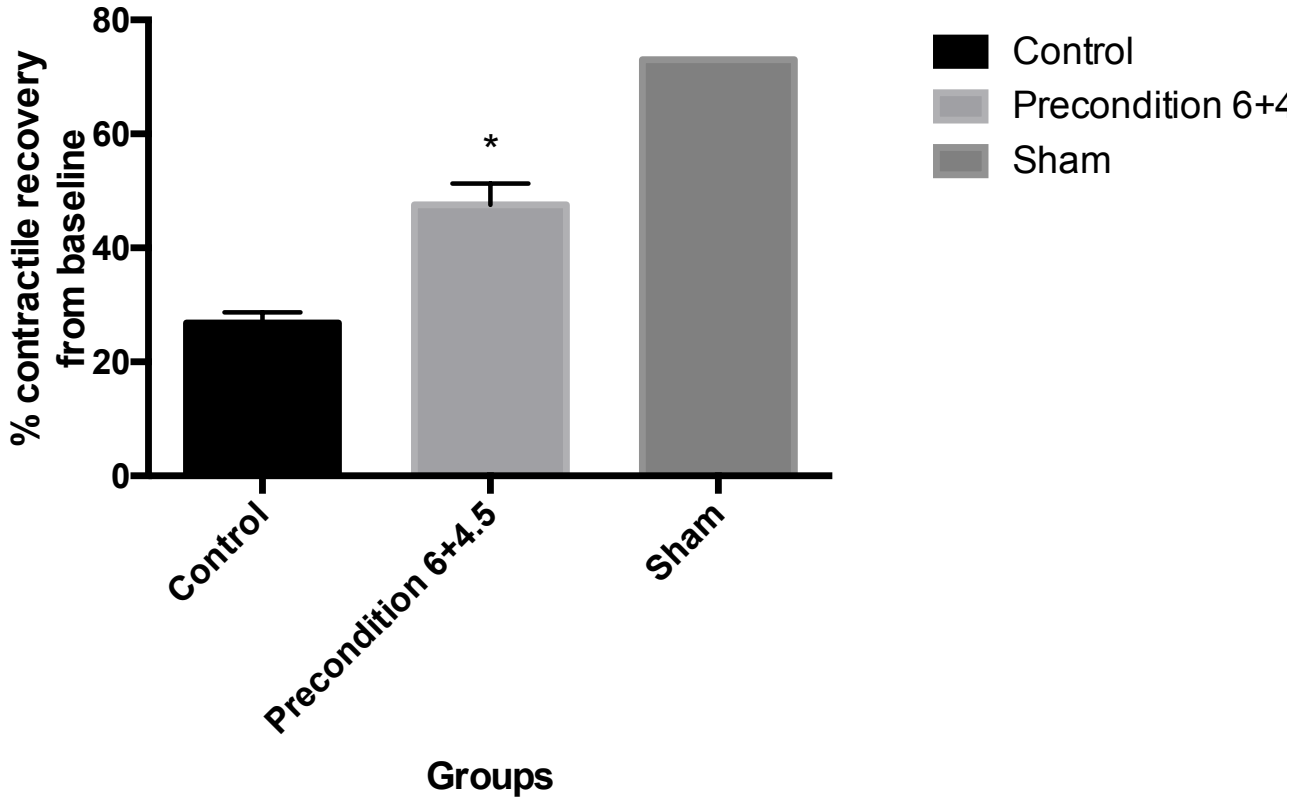


Figure 4.24. Bar chart comparing recovery of function when muscles are subjected to preconditioning 6+4.5 minutes

Chapter 5: Investigating the role of SDF-1α in the protection of human muscle from hypoxia-reperfusion injury

5.1 Introduction

Numerous studies have confirmed that the chemokine SDF-1α confers myocardial protection from the sequelae of lethal reperfusion injury resulting from reperfusion therapy during an MI. The effect of SDF-1α has only been studied in animal models such as mice (181) where it has been to show to modulate IRI in the acute phase and recruit stem cells in the longer term. Whether or not SDF-1α confers the same cardioprotection in human myocardium is yet to be elucidated. This unique study was the first of its kind and was aimed at evaluating the protective potential of SDF-1α.

5.2 Hypothesis Two

Human atrial trabeculae can be chemically preconditioned using SDF-1α through the CXCR4 receptor.

5.3 Study subjects

There were 41 patients included in this set of experiments. All patients met the inclusion criteria as described earlier. The table below outlines the profile of patients that were included in this part of the study.

Profile		Value
Age		**63.2 y**
Gender	**Male**	**35**
	Female	**6**
Ethnicity	**White**	**29**
	Asian	**12**
Type of surgery	**Bypass only**	**23**
	Valve only	**4**
	Combined	**14**
Previous MI		**6**
Previous coronary intervention		**6**

Table 5.1. Patient profile for hypothesis two.

In addition the proportion of patients on the common cardiac drugs were; aspirin (90%) statin (63%) angiotensin converting enzyme inhibitor (40%) and β-blocking drugs (38%). To minimise intraoperative bleeding risk, aspirin was discontinued at least 3 days before surgery. A large proportion of patients were on at least dual anti-angina therapy (β-blocker and calcium channel antagonist) as would be expected in patients undergoing coronary artery bypass surgery. In some cases patients were not entirely sure of their exact drug history therefore details were obtained from their most recent clinic letter.

5.4 Materials and method

5.4.1 Human SDF-1α

Human SDF-1α (research grade) was obtained from Miltenyi Biotec, Germany. They were supplied as 100mcg vials and produced in E.Coli. The ED_{50} according to the manufacturers was between 5-50ng/mL. A working concentration of 25ng/ml was chosen based on previous experimental data obtained from animal models (271, 272). As per manufacturer instructions, reconstitution was suggested with deionized sterile-filtered water to a final concentration between 0.1-

1.0mg/ml in a minimal volume of 100µl with further dilution to take place with either 0.1% bovine serum albumin or human serum albumin in phosphate-buffered solution.

In the human atrial trabecular model, SDF-1α was circulated from the smaller 1-litre chamber containing 250ml of modified Tyrodes buffer therefore:

Working concentration = 25 ng/ml = 3.125nM

Stock = 100µg SDF-1α in 1ml distilled water = 12.5µM

In 250ml, 6.25µg (25ng x 250) of SDF-1α would be required.

Therefore for each 100µg vial, 16 experiments (100/6.25) could be conducted.

Using a pipette, 62.5 µl of the stock solution was transferred to aliquots and stored at -20°C. To eliminate any errors, only 15 experiments were conducted with each 100µg vial and remainder discarded. During the initial stabilisation phase, the frozen aliquot would be removed from the freezer and left at room temperature to thaw taking around 5-minutes to complete the process. When SDF-

1α was added directly into the organ bath using a pipette, the aliquots were washed out repeatedly with circulating buffer to ensure all the contents were added.

5.4.2 AMD 3100 octahydrochloride

The SDF-1α inhibitor, AMD 3100 (research grade) was obtained from Tocris Bioscience, United Kingdom. They were supplied as 10mg vials. The IC_{50} according to the manufacturer was between 0.02 – 0.13 μM for CXCR4 receptors. A working concentration of 5 μg/ml was based on previous experimental data obtained from animal models (272). As per manufacturer datasheet, reconstitution was suggested with deionized sterile-filtered water and the stock solution stored aliquoted in tightly sealed vials at -20°C or below and used within one month.

In the human trabecular model, AMD-3100 was circulated from the smaller 1-litre chamber containing 250ml of modified Tyrodes buffer therefore:

Working concentration = 5 μg/ml = 6.293μM

Stock = 10mg AMD-3100 in 1ml distilled water = 12.6 mM

In 250ml, 1.2mg (5 μg x 250) of AMD-3100 would be required.

Therefore each 10mg vial, would provide for 8 experiments (10000 μg /1250 μg). Each aliquot contained the exact amount of AMD-3100 for each experiment (125μL of stock solution).

5.5 Objective one

To determine whether SDF-1α protects against simulated ischaemia-reperfusion injury in human myocardium

5.5.1 Experimental protocol

This study evaluated the effect of SDF-1α given 30 minutes prior to the index hypoxia on functional recovery of atrial trabeculae. The dose of 25 ng/ml SDF-1α was chosen based on previous literature that illustrated the effective dose (Huang 2011). The protocols used in this experiment are depicted in figure X and are as follows:

1. **Sham** (n≥1): Atrial trabeculae were allowed to contract for a minimum of 2-hours following the stabilisation phase in the organ bath where they were superfused with oxygenated modified Tyrode's solution and paced at 1 Hz without any further experimental manipulation.

2. **Controls** (n≥11): Atrial trabeculae were subjected to 90 minutes of stabilisation, 60 minutes of simulated ischaemia (index hypoxia) and 60 minutes of simulated reperfusion (reoxygenation)

3. **SDF-1α** (n≥11): Atrial trabeculae were subjected to the same protocol as control with SDF-1α administered 30 minutes prior to index hypoxia (60 minutes into stabilisation). This ensured that all CXCR-4 receptors were saturated.

4. **SDF-1α + AMD-3100 <u>5μg/ml</u>** (n≥4): Atrial trabeculae were subjected to the same protocol as control with AMD-3100 administered 10 minutes prior to the addition of SDF-1α (50 minutes into stabilisation). This ensured that all CXCR-4 receptors were antagonised.

5. **SDF-1α + AMD-3100 <u>10μg/ml</u>** (n≥10): Atrial trabeculae were subjected to the same protocol as control with AMD-3100 administered 10 minutes prior to the addition of SDF-1α (50 minutes into stabilisation). This ensured that all CXCR-4 receptors were antagonised.

6. **AMD-3100** (n≥4): Atrial trabeculae were subjected to the same protocol as control with AMD-3100 administered 50 minutes into stabilisation.

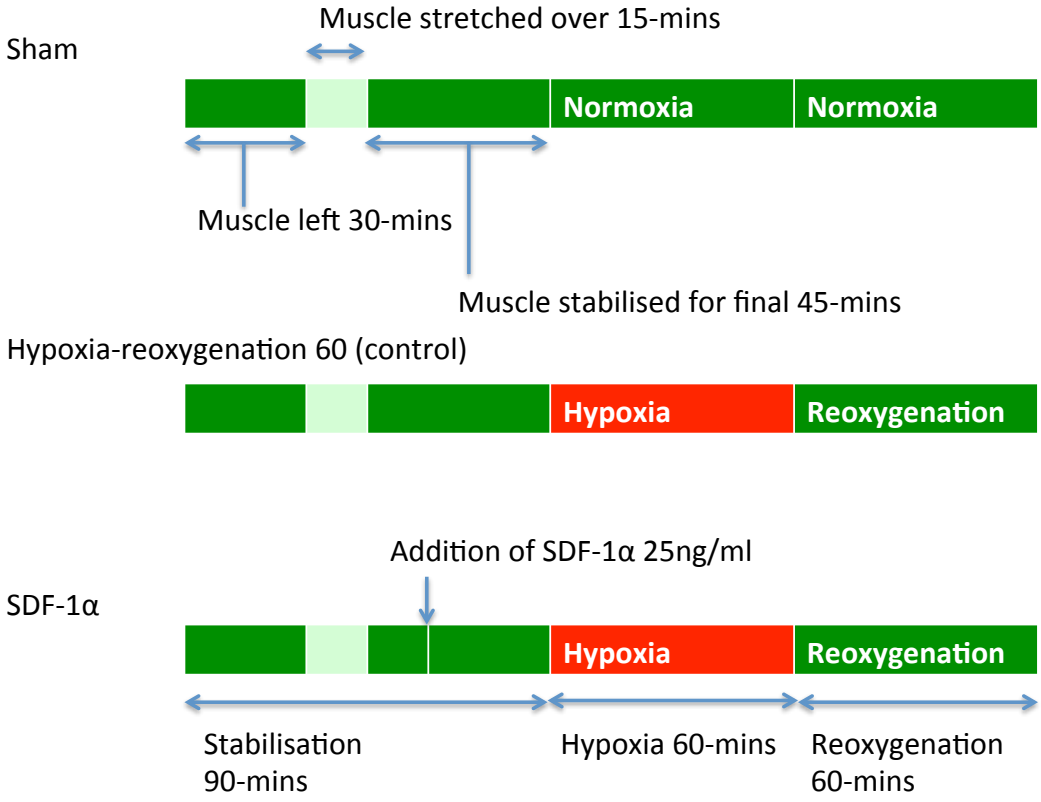


Figure 5.1. Protocols used in SDF1α experiments. Vertical arrow represents point at which 25ng/ml SDF1α was added during the final stabilisation phase.

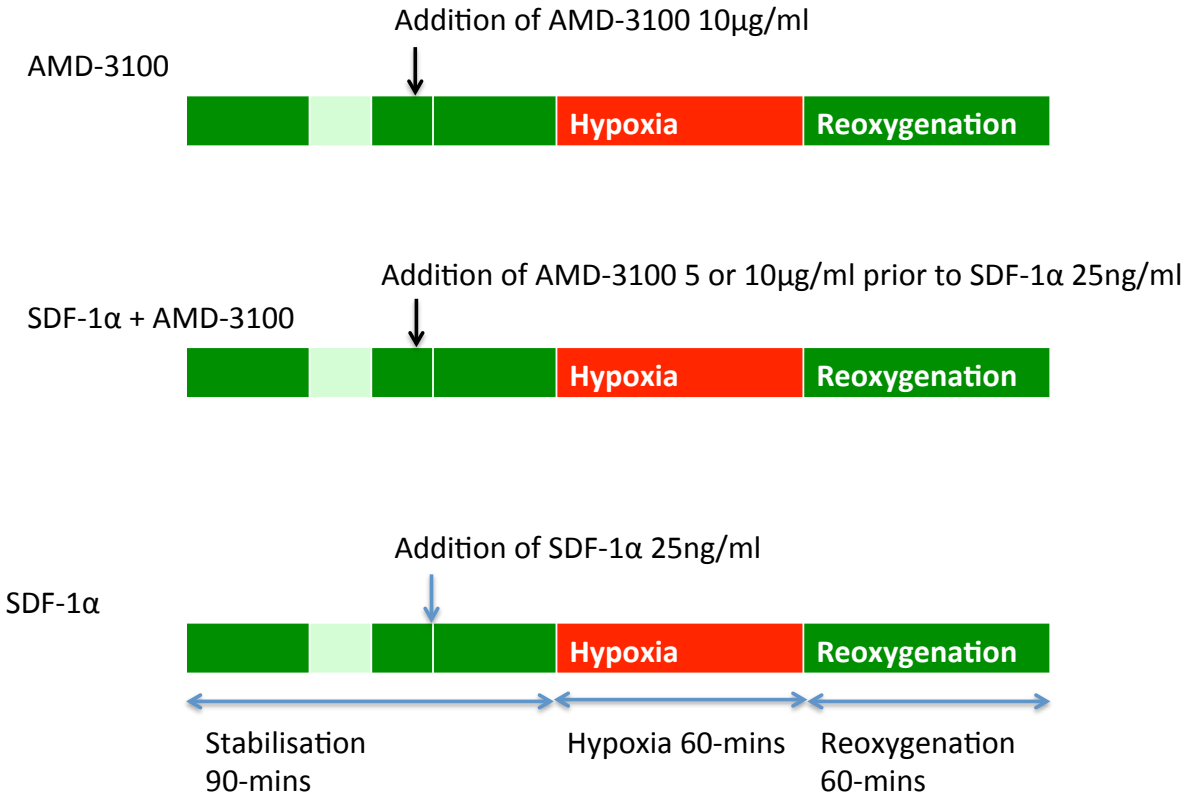


Figure 5.2. Protocols used in SDF1α experiments. Vertical arrow represents points at which 25ng/ml SDF-1α or AMD-3100 was added during the final stabilisation phase.

5.5.2 Results

5.5.2.1 Patient profile

A total of 41 patients were included in this set of experiments, 6 female and 35 male. The ages ranged from 45 to 79.

5.5.2.2 Baseline functional data

Trabeculae in all groups were comparable at baseline in terms of average baseline force of contraction as shown in the table below. Baseline data was collected at the end of the 90 minutes stabilisation period.

Group	**Number**	**Average contraction in grams)**
Sham	**1**	**1.50**
Control	**11**	**0.75±0.06**
SDF1α	**11**	**1.10±0.12**
SDF1α + AMD 5μg	**4**	**1.16±0.23**
SDF1α + AMD 10μg	**10**	**0.85±0.05**
AMD 10μg	**4**	**0.96±0.07**

Table 5.2. Baseline data for objective one.

Table 5.3. Raw data for control only experiments.

Experiment	Peak force (g)	Recovered force (g)	Trabecular length (mm)	Functional recovery (%)
1	0.87	0.22	3.06	24.91
2	0.81	0.20	3.06	25.20
3	1.14	0.31	4.38	27.50
4	0.85	0.22	4.38	25.33
5	0.50	0.11	3.68	22.53
6	0.85	0.28	4.8	32.57
7	0.62	0.20	4.4	32.06
8	0.77	0.27	5.3	35.42
9	0.50	0.14	3.9	28.44
10	0.63	0.18	4.4	28.61
11	0.72	0.10	3.5	12.82

Table 5.4. Raw data for SDF-1α only experiments.

Experiment	Peak force (g)	Recovered force (g)	Trabecular length (mm)	Functional recovery (%)
1	1.55	0.93	7.00	60.00
2	1.33	0.66	3.40	49.48
3	0.55	0.22	4.20	39.47
4	1.08	0.49	5.20	45.25
5	0.68	0.41	4.40	60.50
6	0.54	0.39	7.9	71.87
7	0.70	0.35	3.50	49.38
8	1.00	0.44	6.20	44.64
9	1.65	0.97	6.13	58.83
10	1.47	0.77	4.99	52.72
11	1.22	0.62	2.63	51.40

5.5.2.3 Recovery of function

Preconditioning with SDF-1α

SDF-1α administered 30 minutes prior to index hypoxia at a concentration of 25ng/ml resulted in a functional recovery of 53.1% ± 2.8%. This was significantly higher than that of the control group, 26.9% ± 1.8%, with a p-value of <0.05. The positive effects of SDF-1α were blocked with the addition of AMD-3100 10μg/ml 10 minutes prior to SDF-1α (29.6 ± 2.4%) but not with the lower dose of AMD-3100 5μg/ml (54.0 ± 6.4%). AMD-3100 10μg/ml had no effect when given alone (21.3 ± 2.7%).

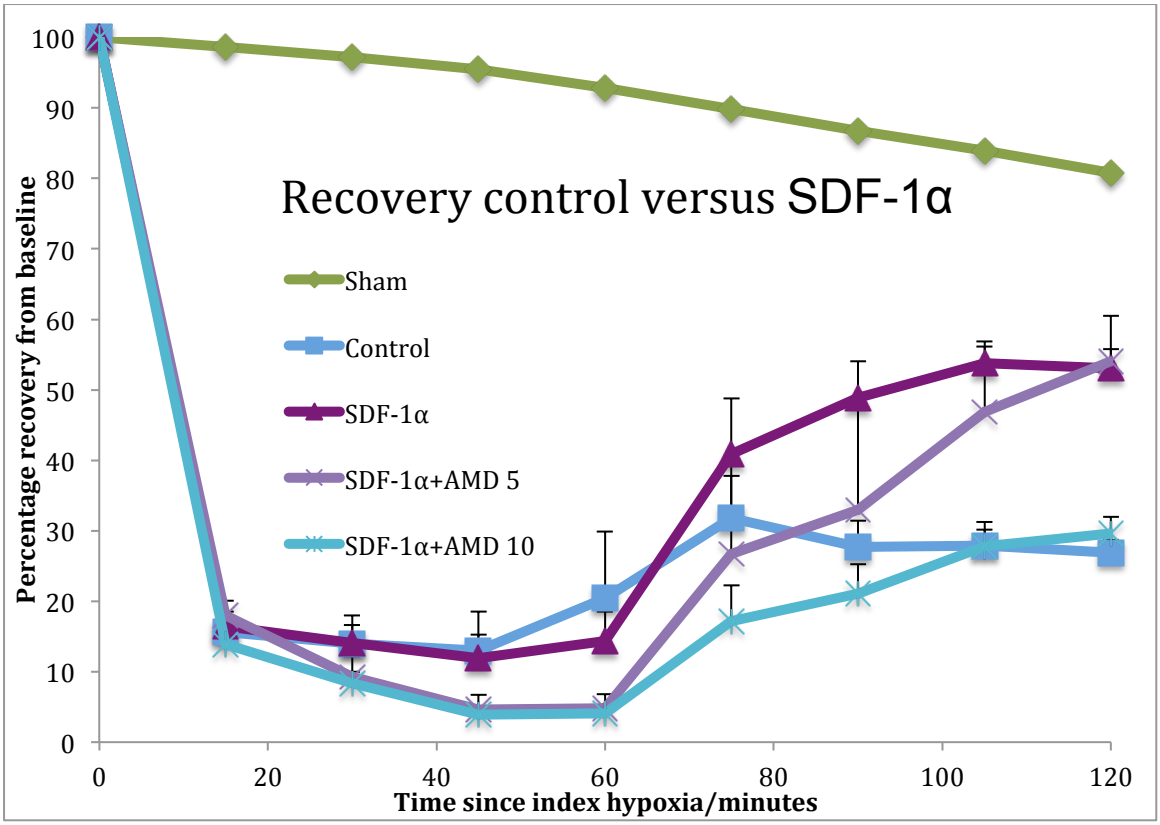


Figure 5.3. Comparison of recovery of function for various groups

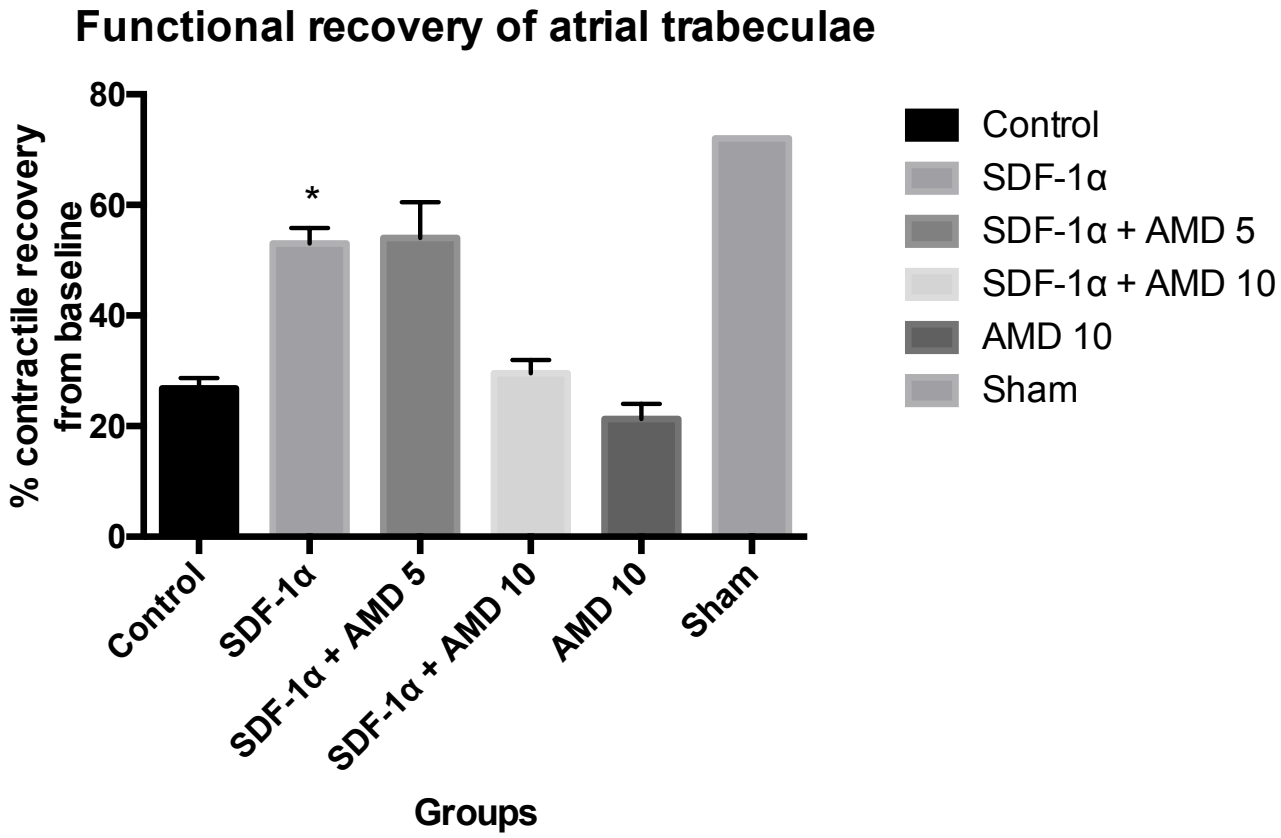


Figure 5.4. Bar chart representing recovery of function at 60 minutes since reoxygenation.

5.6 Objective two

To confirm the presence of CXCR4 (SDF-1α) receptors on human atrial trabeculae.

5.6.1 Experimental protocol

As mentioned previously Dr Jose Vincencio conducted this part of my study. The purpose was to confirm the presence of SDF-1α receptors on human tissue. The method is described in chapter 3, section 3.14 immunuhistochemistry. Rat and mice whole heart and cardiomyocyte (CM), are known to contain CXCR4 receptors were used as a positive controls.

5.6.2 Western blots

The results below are the western blotting for the presence of SDF-1α in human tissue. This was performed on human CM isolated from atrial trabeculae. Please note that western blotting was also performed on rat CM, whole rat and mice heart.

Figure 5.5. Western blotting for human CXCR4 receptor.

5.6.3 Immunofluorescent staining

The result below (upper 3 panels) demonstrates the distribution of CXCR4 (green) in relation to cardiomyocyte (red). The lower 3 panels demonstrates immunofluorescent staining using secondary antibody in the absence of primary antibody. Representative images from *n=2* independent experiments, 50 μm scale bar.

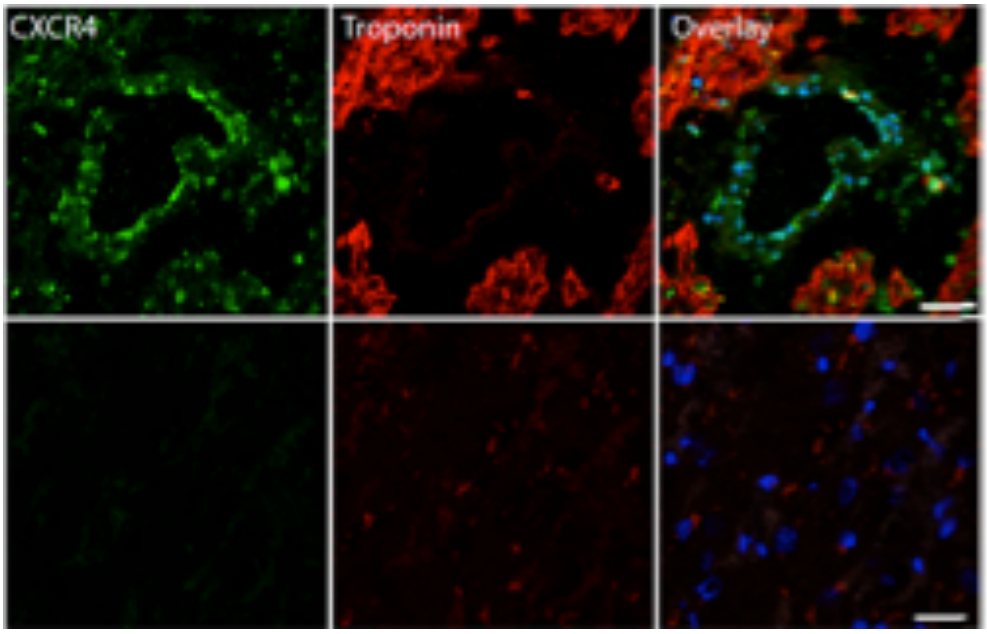


Figure 5.6. Immunofluorescent staining for human CXCR4 receptor.

5.7 Objective Three

To confirm SDF-1α confers protection via activation of the RISK pathway.

5.7.1 Experimental protocol

As mentioned earlier Dr Jose Vincencio conducted this part of my study. The purpose was to confirm the activation of the RISK pathway. The method is described in chapter 3, section 3.14 immunuhistochemistry. Insulin, which is known to activate Akt and Erk was used as a positive control.

5.7.2 Western blots

The results below are the Western blotting demonstrating the activation of RISK pathway.

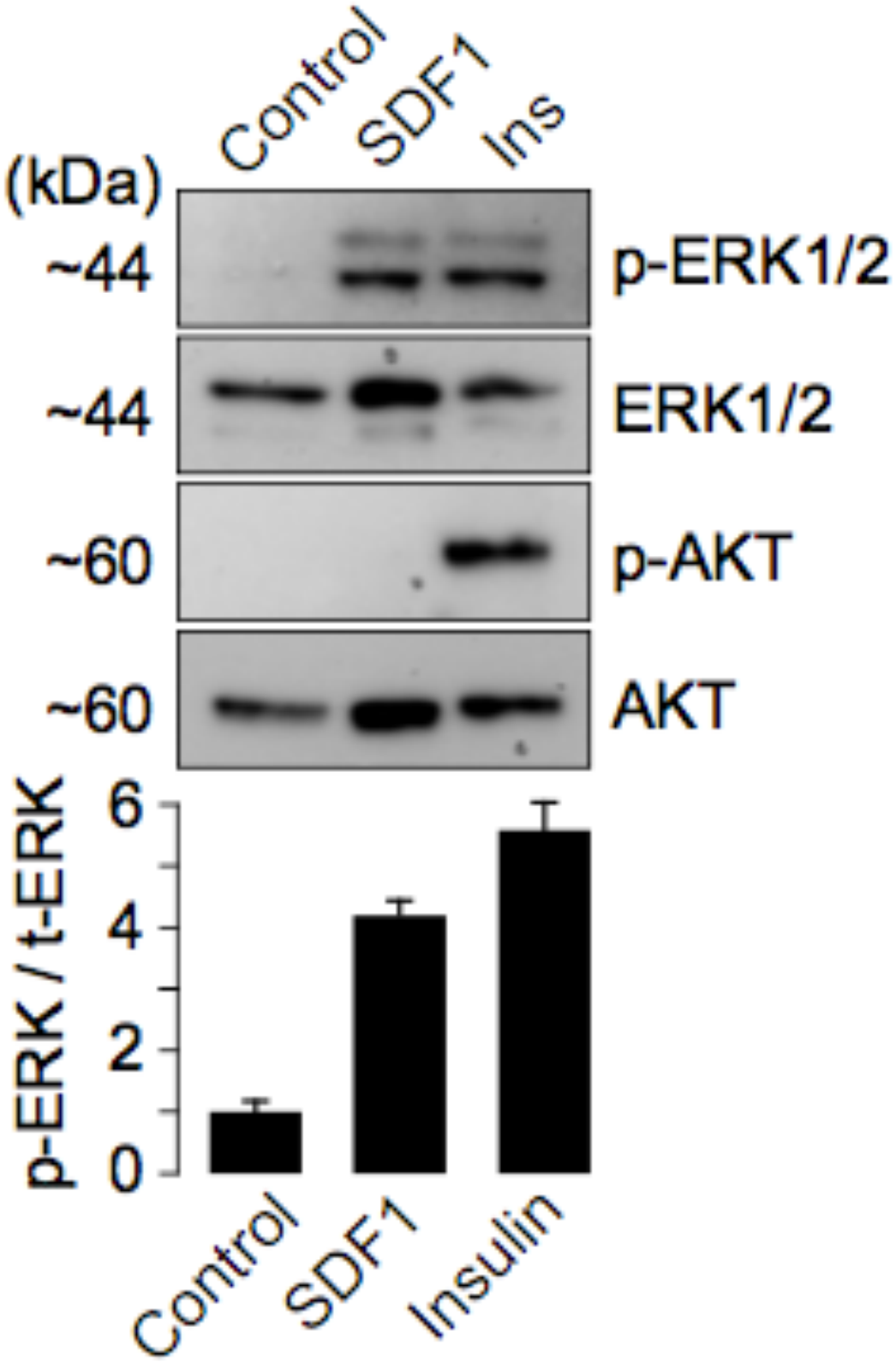


Figure 5.7. Western blotting for the activation of the RISK pathway.

CHAPTER 6: Discussion

6.1 Summary of this study

The present study confirms (1) the presence and location of SDF-1α receptors (CXCR4) in human adult cardiomyocytes (2) the ability of SDF-1α to attenuate the detrimental effects of IRI on human tissue, (3) using the specific receptor blocker, AMD3100, can attenuate benefits conferred by chemically preconditioning by SDF-1α, (4) AMD3100 itself has no effect on the functional recovery of human trabeculae subjected to IRI compared to control, (5) preconditioning effects of SDF-1α could at least be partly explained by activation of prosurvival kinases such as Erk 1/2 and (6) the ability to protect human atrial trabeculae from the detrimental effects of IRI through hypoxic preconditioning.

To the best of our knowledge, this is the first report to illustrate that the SDF-1α – CXCR4 axis in the human atrial trabecular model. A summary graph and bar chart is shown below.

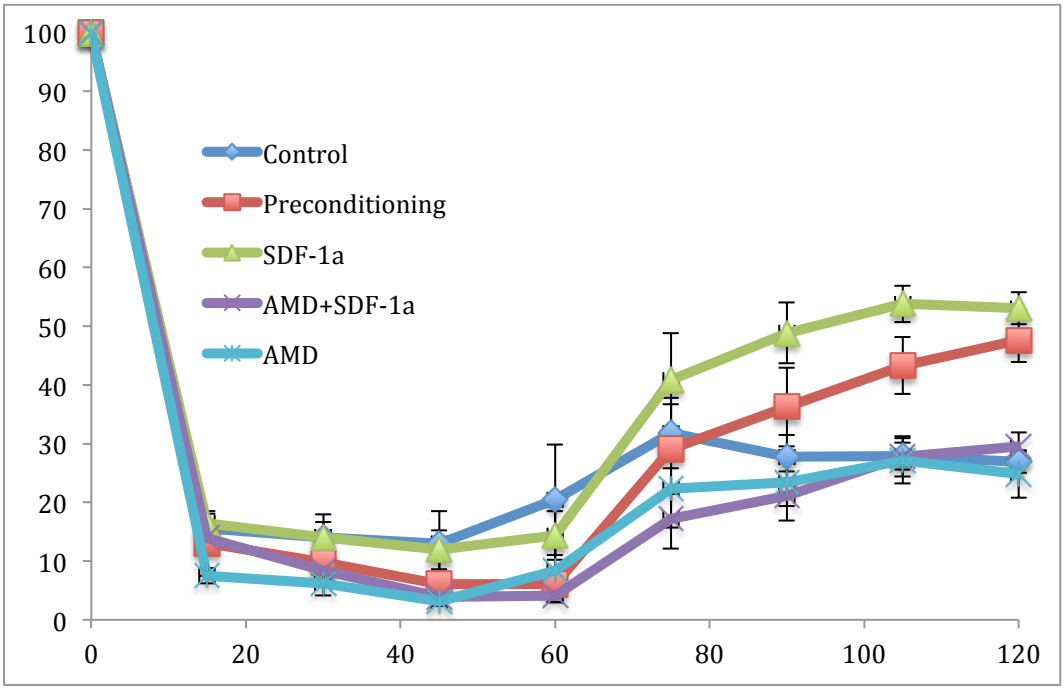


Figure 6.1. Percent recovery of contractile function (Y-axis) during simulated ischaemia and reperfusion.

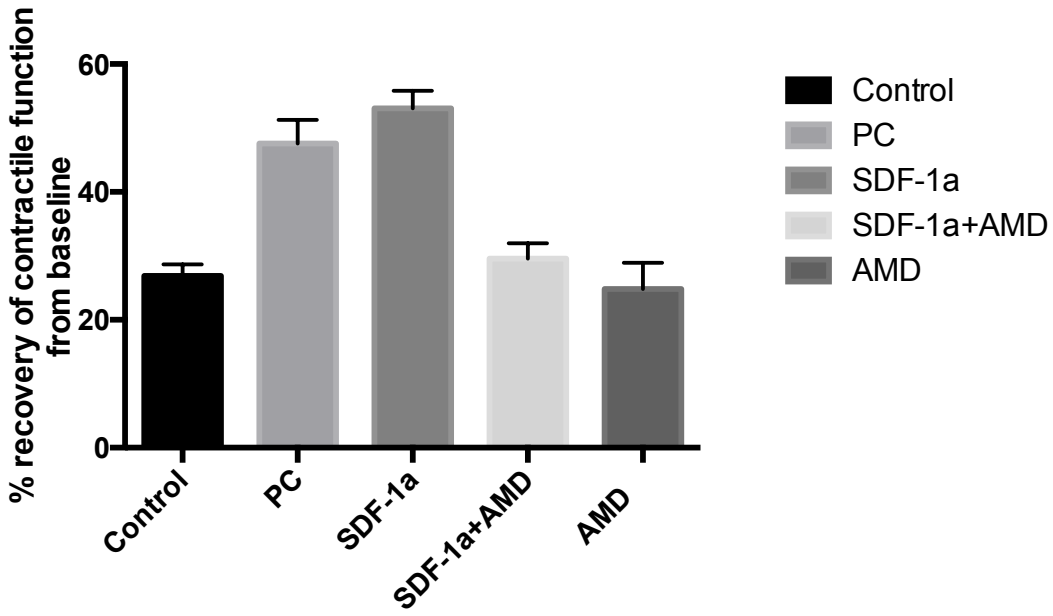


Figure 6.2. Percent recovery of contractile function at 60 mins in various groups.

6.2 Human trabeculae can be preconditioned with simulated ischaemia.

The present study confirms (a) isolated human atrial muscle may be protected from a hypoxic insult (simulated ischaemia) by preconditioning with substrate-free hypoxic Tyrode's solution and rapid pacing, (b) a stabilisation time of 60 minutes is sufficient in the atrial trabecular model of preconditioning and (c) an index ischaemic insult of 60 minutes is adequate to induce injury and also allow a preconditioning protocol to be applied to demonstrate protection i.e. in our study our trabeculae was able to withstand 60 minutes hypoxia and delay cell death induced by hypoxic preconditioning.

There are now several clinical studies that suggest preconditioning occurs in humans, which are described in more detail in the introduction to this thesis in section 1.5.8.

6.3 Difficulties encountered with preconditioning.

During this study it was essential to demonstrate the ability to precondition human atrial trabeculae in order to have a positive control for experiments against lethal reperfusion injury.

Previous investigators at the Hatter Cardiovascular Institute had demonstrated the existence of this in several studies as outlined in the introduction. With advances in clinical pharmacotherapy, many of which have preconditioning properties, it was essential to establish that preconditioning continued to exist in human heart.

Previously the experiments conducted at this Institute consisted of 75 minutes stabilisation time followed by 90 minutes simulated ischaemia with subsequent 2 hours re-oxygenation. Preconditioning applied prior to index hypoxic insult ranged between 3 - 4 minutes of simulated ischaemia followed by 7-16 minutes of reoxygenation. Most authors used 3+7 protocol and were able to successfully precondition human muscle.

In this study, the initial protocol implemented consisted of 90 minutes simulated ischaemia followed by 120 minutes reoxygenation to achieve a functional recovery between 25-35%; this was consistent with previous studies at the Hatter Cardiovascular Institute. However, preconditioning with a protocol consisting of 3 minutes simulated ischaemia followed by 12 minutes reoxygenation based on previous investigators failed to precondition trabeculae. Subsequent protocols with stronger preconditioning stimulus (4+10 and 4+16) also failed to

successfully precondition human muscle. The reason for increasing the stimulus was partly based on previous work on human diabetic heart preconditioning at this Institute where with an established protocol (4+16) failed to precondition muscle and a stronger stimulus (7+16) was required to be able to successfully precondition (267). Besides increasing the preconditioning hypoxia time, the reoxygenation time was also increased as it was felt that the trabeculae may gain benefit by the process of catabolite washing that would have accumulated during the hypoxic episode.

There may be several considerations why it may have been difficult to precondition trabeculae. During recruitment, patients had to meet strict exclusion criteria outlined in section 3.5.1. and trabeculae were excluded according to criteria in section 3.12. Despite reaching the expected baseline contractility of 0.5g it is possible that during the harvesting process, handing of the atrial appendage may have caused mechanical injury that was not apparent macroscopically but somehow prevented them from being preconditioned. Furthermore there was a time delay between harvesting the appendage and isolating the trabeculae to starting the experiment. A solution to overcome time delay

could be to have a laboratory within the hospital next to the cardiac theatres so that harvested appendage could be placed immediately in warm oxygenated buffer and trabeculae isolated with minimal interruption. Transport to the laboratory from the hospital is likely to incur some thermal injury with cooling down to 4°C which is necessary to reduce the metabolic rate of the cardiomyocytes.

Other important considerations are the diabetic status and drug history of the patients. It has been established that diabetic patients require an enhanced preconditioning stimulus to be preconditioned; this is at least partly due to down-regulation of the PI3K-Akt signalling pathway (267). Many patients recruited in this study were not diabetic as this was an exclusion criterion (section 3.5.1). However, with a high prevalence of undiagnosed diabetes in the UK, it is possible that patients were not aware of their glycaemic status. Also no formal tests were carried out to exclude this. With increasing prevalence of diabetes, this partly may explain why the trabeculae in this study failed to get preconditioned with an established standard protocol but required a higher stimulus. It may have been a useful exercise to exclude diabetes biochemically prior to recruitment into study.

Many patients recruited in this study were on several cardiac drugs that may have a direct preconditioning effect. These drugs include statins, beta-blocking drugs and ACEi. In an isolated atrial trabecular model by Dr Rees at the Hatter Cardiovascular Institute, high dose atorvastatin administered 12 hours before cardiac surgery has been shown to improve functional recovery when compared to patients on chronic statin therapy (273). It is known from earlier studies that bradykinin is a trigger for preconditioning (274). In a subsequent experiment by Morris et al, pretreatment with an ACEi combined with a subthreshold preconditioning stimulus lead to an improved function recovery in isolated human atrial trabeculae as seen with a standard preconditioning stimulus (108). Other drugs that have preconditioning effects would include opioid analgesia given at anaesthetic induction – this has also been shown in a human atrial trabecular model to precondition muscle (133). Similarly anaesthetic preconditioning with volatile anaesthesia is a well-described phenomenon and has been shown to provide protection against ischaemia-reperfusion injury (275). All patients recruited in this study were subjected to volatile anaesthesia. If these drugs were to precondition the heart then the functional recovery

following standard hypoxia-reoxygenation protocol would have been higher, which was not the case in this study. An argument against this could be that the beneficial effects of these agents may not have been apparent as in classical preconditioning, there is a temporal relationship between the preconditioning stimulus and index hypoxia. (1st window of PC has passed). Blockers of preconditioning include sulphonylureas (diabetic drug), which does not apply in this study. Briefly it is thought to block preconditioning by blocking ATP-sensitive K^+ channels (K_{ATP} channels) (106-107).

Given the inability to precondition human tissue, the next part of the study was therefore to re-characterise the human trabecular model despite its use since 1990's. The first characterisation step established whether a stabilisation time of 90 minutes was necessary. This was evaluated by assessing the ratios of activated Akt or Erk to total Akt or Erk. Insulin was used as a positive control as it has been established to activate both these kinases (276). Finding from this part of the study suggested high ratio of kinases at 10 and 30 minutes with a reduction at 60 minutes. There was no significant difference at 120 minutes. At 60 minutes, addition of insulin increased kinase ratios confirming

there was still capacity for muscle to be preconditioned. Therefore a stabilisation time of 90 minutes was chosen to allow muscle to settle for at least 45 minutes following achievement of the peak of the Frank-Starling curve.

We then investigated different hypoxia times. Although a hypoxia time of 90 minutes was achieving a functional recovery similar to previous investigators, it may have been that this exceeded the level of damage caused to cardiomyocytes to enable them to be preconditioned. 90 minutes hypoxia times may have caused so much damaged to the CM that the efficacy of PC was diminished. Index hypoxia times of 45 and 60 minutes were chosen. With a 45 minutes hypoxia time, insufficient muscle injury was caused therefore a longer hypoxia time of 60 minutes was trialed. This produced similar injury levels to that of 90 minutes hypoxia therefore a hypoxia time of 60 minutes were taken as part of the protocol. What has been established from Murrys original study is that preconditioning act to delay rather than prevent cell death. In their earlier observation, rate of ATP depletion was equivalent in control and preconditioned cell but cell death was reduced in the preconditioned cells implying they were more resistant to the detrimental effects of hypoxia. However, when a prolonged

ischaemia time was applied, the benefits of preconditioning were abolished confirming preconditioning acts to delay cell death. What is also known from previous studies that there is a temporal aspect to preconditioning; that is, there is a limit to how much ischaemia cells may resist or withstand. Murrys original canine study consisted of 40 minutes circumflex occlusion followed by 4 days of reperfusion. Nao et al (80) demonstrated this in a similar canine model to Murry where the protection conferred by preconditioning was maintained with 60 minutes of coronary occlusion but lost with 90 minutes of occlusion. This may indeed be one of many reasons why in this study, human muscle failed to be preconditioned with 90 minutes hypoxia.

The next question was whether the gradual stretching during the stabilisation period affected the ability to precondition isolated human trabeculae. Dr Rees established a similar model at the Hatter Cardiovascular Institute where a single stretch was applied at the outset of the experiment and left to stabilise before protocols were applied. This was the case irrespective of the length or width of trabeculae. A criticism was how the same force can be applied to different trabeculae and how would one ensure the peak of the Frank-Starling curve was achieved? What is known

is overstretching atrial muscle could have a negative inotropic effect (270). Bearing this in mind, a comparison was made between multiple stretches and minimal stretches (that consisted of a stretch at the beginning and again 20 minutes into the experiment). The finding from this part of the study confirmed there was no significant difference in functional recovery between the control and preconditioned (4+16) trabeculae whether they were stretched multiple times or minimally. These findings contradict earlier studies on stretch-induced preconditioning. Ovize and colleagues were the first authors to propose that preconditioning triggered by brief ischaemia lead to transient stretching or dilatation (stretching), questioning whether if it was the effect of stretch that induced the protective effects rather than ischaemia per se. This was confirmed in their canine model where acute volume overloading their heart leads to reduced infarct sizes. Furthermore they proposed that the protection afforded by stretch was mediated by stretch-activated channels that was abolished in the presence of Gd3+, which is a potent blocker of stretch-activated channels (254). Nakagawa et al used an isolated perfused heart Langendorff model to demonstrate that stretch (a form of non-ischemic stress), induced by transiently increasing

left ventricular end diastolic pressure (LVEDP) for 5 minutes preconditioned rats hearts as demonstrated by improved haemodynamic parameters of LVEDP and left ventricular developed pressure (LVDP). Importantly coronary blood flow did not alter during the increase in LVEDP hence the term 'non-ischaemic stress' (277). In another study by Gysembergh et al, an in-vivo rabbit model was used to demonstrate preconditioning by stretch where this was induced by transient volume overload. The same authors went on to describe the similarities in mechanistic that exist between ischaemic preconditions and stretch preconditioning such as downstream activation of PKC, adenosine receptors and/or K^+ATP channels (107).

There may be several explanations why stretching the trabeculae did not precondition the heart; the studies mentioned above involve whole hearts compared to isolated atrial trabeculae used in this study. Secondly the stretch induced was rapid; in the study by Nakagawa et al (253), LVEDP was rapidly increased from a baseline of 10 mmHg to either 30 or 60 mmHg. In comparison, the trabeculae were stretched gradually over 15 minutes to the peak of the Frank-Starling curve with each stretch with the micromanipulator not exceeding a force of 0.1g and 0.05g towards

the peak. It could be that a sudden large stretch is necessary to activate the stretch-activated channels. This could be related to work by Downey and colleagues (274) who proposed that there was a summative interaction of preconditioning triggers such that a particular threshold must attained to achieve analogous to 'all or none' phenomenon seen with action potentials. This concept has been illustrated in this laboratory using an isolated atrial trabecular model where two separate stimuli (elevated bradykinin by ACEi or sublethal hypoxia) were inadequate to provoke a preconditioning response when administered separately, but in combination, a full protective response was detected (108).

Finally, having established an appropriate stabilisation and hypoxia time, preconditioning was re-attempted. This consisted of a protocol consisting of 6 minutes hypoxia and 4.5 minutes reoxygenation. With the re-characterised human trabecular model, it was possible to demonstrate the successful preconditioning of isolated human atrial trabeculae. Now that the model was accomplished, attention was focused on SDF-1α.

6.4 Human trabeculae can be chemically preconditioned with SDF-1α.

These experiments demonstrate that SDF-1α protects human tissue from lethal reperfusion injury. The key findings in this part of the study are as follows: (1) the presence and location of SDF-1α receptors (CXCR4) in human adult cardiomyocytes (2) the ability of SDF-1α to attenuate the detrimental effects of IRI on human tissue, (3) using the specific receptor blocker, AMD3100, can attenuate benefits conferred by chemically preconditioning by SDF-1α, (4) AMD3100 itself has no effect on the functional recovery of human trabeculae subjected to IRI compared to control and (5) preconditioning effects of SDF-1α could at least be partly explained by activation of prosurvival kinases.

In this study we demonstrate that SDF-1α at 25 ng/ml, for the first time, protects human atrial trabeculae subjected to simulated ischaemia-reperfusion injury. This finding is consistent with published data obtained from animal models where SDF-1α has been shown to confer myocardial protection in MI related to the modulation of ischaemia-reperfusion injury rather than stem cell recruitment (278). Saxena et al demonstrated intracardiac injection of SDF-1α into the myocardium adjacent to the ischaemic zone at the

time of ligation of left anterior descending artery in mice hearts resulted in significantly better cardiac function after an MI through activation of cell survival kinase, protein kinase B/Akt within endothelial cells and myocytes of mice hearts (247).

In a separate murine model of ischaemic preconditioning, Hu and colleagues were able to demonstrate increased levels of myocardial SDF-1α mRNA (cardiac myocytes and fibroblasts). In isolated myocytes, increased phosphorylation of both Erk 1/2 and Akt and decreased phosphorylation of JNK and p38 were noted when CXCR4 receptors were activated with SDF-1α (243).

It is important to understand that pre-ischaemic exposure of SDF-1α to the myocardium, as was the case with this study, is less clinically relevant unless ischaemia is planned such as during cardiac surgery or transplantation. However there have been studies that have examined the role of SDF-1α targeting specifically the reperfusion phase i.e. post-ischaemia that would be more clinically relevant following an MI (278). In this experimental study by Jang et al, Langendorff isolated rat hearts infused with varying concentrations of SDF-1α 10 mins prior to reperfusion reduced the area of necrosis relative to the area at risk in a dose dependent manner. This was abolished in the presence of AMD-3100 given 10 minutes prior to

SDF-1α. Similar results were noted with pre-ischaemic exposure to SDF-1α. Interestingly, authors noted the infarct sparing effect was greater than that seen with ischaemic pre and post-conditioning. These findings are similar to this study where pre-ischaemic exposure to SDF-1α lead to improved functional recovery and effects abolished when AMD-3100 was administered 10 minutes prior to SDF-1α.

Another important finding in the present study is the phosphorylation of Erk 1/2 by SDF-1α in cardiomyocytes. This again is consistent with many experimental finding such as work done by Hu et al noted an increase in phosphorylation of Erk 1/2 and Akt in isolated rat cardiomyocytes when CXCR4 receptor were activated with SDF-1α (221). In a separate study by Jang et al, the effect of SDF-1α on myocardial protection during the reperfusion phase was studied. This was performed using a Langendorff model where SDF-1α was added to the reperfusion buffer following LAD ligation in rat hearts to demonstrate a reduction in area of necrosis: area at risk in a dose dependent manner. Furthermore, the involvement of Akt and Erk were demonstrated during post conditioning of rat hearts (278). Interestingly, Akt phosphorylation was not demonstrated with SDF-

1α in cardiomyocytes, however, this cannot be completely ruled out without further experiments. More importantly, inter-specie variation may explain these results.

6.5 Critique of atrial trabecular model.

Critical analysis of the superfusion model is mentioned in section 3.2. Additionally it is important to discuss whether hypoxia is as effective as ischaemia and whether improved functional recovery equates to a reduction in cell death.

6.5.1 Effectiveness of hypoxia at inducing preconditioning compared to true ischaemia.

Important differences exist between true ischaemia seen in real life compared to experimental models where in this study hypoxia or 'simulated ischaemia' is emulated. In our study this was achieved by using hypoxic substrate free buffer in conjunction with rapid pacing.

The degree of metabolite accumulation in true ischaemia is greater than that observed in experimental models and the pH change may not be to the same degree (279). Therefore it may not be possible to

extrapolate the findings from this study where preconditioning was produced by simulated ischaemia to circumstances of preconditioning with ischaemia. However, there is now a wealth of evidence from experimental models that simulated ischaemia is as effective as ischaemia in eliciting protection. These include models consisting of cell culture models, global and regional ischaemia. To illustrate this, Lasley et al demonstrated the comparable effectiveness of preconditioning using ischaemia and hypoxia in a Langendorff model using rat hearts where the hearts were subjected to either 5 minutes of hypoxia or ischaemia. Lasley observed the same post-ischaemic functional recovery in both groups and proposed that reduced oxygen delivery to the tissue was more important than metabolite accumulation to induce preconditioning. In a similar model by Engleman et al, the effect of preconditioning with hypoxia was compared against rat hearts without preconditioning on cell necrosis (assessed by LDH release) and functional recovery. Preconditioning with 10 minutes of hypoxia ensued a reduction in cell necrosis and improved functional recovery suggesting hypoxia is an effective preconditioning stimulus (280). Using a cell culture model, Webster et al have also demonstrated the

ability to successfully precondition neonatal rat myocytes using hypoxia (281).

6.5.2 Improvement of functional recovery equating to reduced cell death.

This study uses force of contraction as a measure and recovery of function as a surrogate marker of muscle injury. It does not measure the degree of necrosis. Experimental protocol used in this study were as long as 230 minutes. It is probable that there was an area of central necrosis with contractions being due to the outermost cardiomyocytes and by the end of the protocol there is almost certainly likely to be a further reduction of viable cells. Therefore further experiments such as Western blotting to evaluate survival kinases is difficult, thus this model provides only indirect evidence through functional recovery in relation to preconditioning. However, other in vitro experiments by Jenkins et al have demonstrated an infarct sparing effect with preconditioning where improvement of global left ventricular function mediated through preconditioning was proportional to a reduction in infarct volume (282). Furthermore earlier investigators including Pryzklenk et al (79) and

Cohen et al (75) were able to associate improved recovery of systolic shortening to a reduction in infarct size in an in vivo model of regional ischaemia. To date there is no direct evidence in an isolated trabecular model that improved functional recovery is related to a reduction in infarct volume but given the body of evidence, it is likely to be the case.

Given the duration of the simulated ischaemic insult of 60 minutes in this study, myocardial necrosis is more likely than stunning that is usually induced by shorter periods of ischaemia (5-15 minutes) confirming this is a model of preconditioning. As with any scientific conclusion, it would be difficult to say that this model does not involve myocardial stunning because Auchampach et al have shown that potassium channel openers are efficacious in inducing protection in canine models of both stunning and infarction (283).

6.6 Clinical studies of SDF-1α and cardioprotection.

Based on some of the previous evidence discussed in this thesis, the advantages of manipulating the SDF-1α/CXCR4 had proven beneficial only in animal based experimental studies.

At present, to the best of my knowledge, there are no clinical trials exploring the role of SDF-1α in the context of a myocardial ischaemia-reperfusion injury model. Kim et al have assessed the release of SDF-1α in cardiac surgery patient and have noted their release specifically during myocardial ischaemia but not during reperfusion whilst also noting an inverse relationship between SDF-1α release and organ dysfunction (284).

Investigators have tried to manipulate the SDF-1α/CXCR4 axis at several points in the cascade including through inhibition of dipeptidyl-peptidase-4 inhibitors, which is a class of drugs shown to be effective for the treatment of type II diabetes mellitus (285). SDF-1α is also known to be a substrate for this enzyme therefore by inhibiting the breakdown of SDF-1α, it was thought this may have a positive effect on cardiovascular outcome. As such, several outcome studies were designed to evaluate the effect including EXAMINE, TECOS and SAVOR-TIMI trials (286-288). The major endpoints for these studies were major adverse cardiovascular events (MACE) in patients who were already at high risk for cardiovascular disease (diabetics, recent coronary events). None of these studies demonstrated an increase in cardiovascular outcomes but patients on saxagliptin did have a higher incidence of hospitalization for heart

failure, for reasons that remain unclear. Importantly, there was not a reduced MACE in cohort of patients studied.

6.7 Conclusion

The present study demonstrates that preconditioning continues to be effective in human tissue even after 3 decades since the initial discovery, despite advances in medical therapy that have important preconditioning effects. SDF-1α/CXCR4 axis may play a central role in preconditioning the myocardium in human and other species. It demonstrates that preconditioning may be mediated through activation of Erk 1/2 kinases that has been shown in all other forms of conditioning. With limited numbers of patients recruited in this study, the findings must be reviewed with this in mind. This study is the first to demonstrate that SDF-1α has the ability to precondition human myocardium.

With experimental evidence available in animal models, the ultimate goal of this study was to determine whether preconditioning with SDF-1α in human tissue was possible and also to begin to understand mechanisms involved so that one day drug therapy may be developed to minimise the insult to the heart from the detrimental

effects of a myocardial infarction. The findings in this study add new information to the field of preconditioning that may bring closer the search for a novel therapeutic strategy.

6.8 Future directions

Given the challenges encountered during this research period for preconditioning, it would be suggested that future patients recruited should all have biochemical exclusion of diabetes mellitus.

If time was permitted, it would also be interesting to explore whether with the optimal stabilisation and hypoxia times, atrial trabeculae could be preconditioned with the same preconditioning protocol used earlier that failed with the longer duration of hypoxia and reoxygenation times.

Only a single dose of SDF-1α was used based on published experimental studies. It would be valuable to perform a dose response curve in human tissue with SDF-1α as well as AMD-3100.

Finally, the use of specific inhibitors of the RISK pathway in the presence of SDF-1α to demonstrate protection is mediated (at least partially) through this pathway would be a valuable demonstration.

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Appendix

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Version 2 July 2007

PATIENT INFORMATION SHEET-CONFIDENTIAL

An investigation into the effects of potential cardioprotective therapies on human heart muscle

We are undertaking research to identify agents that will have a significant effect of protecting the heart during coronary artery bypass graft surgery or limit the damage caused by a heart attack. Research has shown that it is possible to protect animal hearts from the damaging effects of a heart attack using a variety of interventions. In contrast however there is little experience in man. We seek to investigate whether protection can occur using samples of human heart muscle.

What does the study involve?

We wish to collect pieces of human heart muscle (the right atrial appendage) that can be harvested as part of the process of establishing cardiopulmonary bypass. This muscle can then be used in a number of experimental models that can mimic what happens during a heart attack. Using various drugs that may protect the heart, we will then be able to look for improvements in outcome that could eventually be clinically relevant.

During the Operation

Your heart operation will proceed in the usual way. During your operation and at the time that the pipes of the bypass machine are inserted into the heart, a small piece of heart muscle will be removed, a procedure which is not harmful. This small piece of heart muscle will be used in our laboratory investigation to investigate the effects of agents that can offer the heart significant protection.

NB This study has been approved by the hospital Ethics Committee.

Are there any risks involved?

Your surgeon and his team will have explained to you that there are risks involved with any heart surgery. The harvesting of the right atrial appendage is not harmful and entails no further risk.

Are your results confidential?

Only the doctors and research workers in this hospital will see your results. Your individual details will not be revealed if the findings are presented at meetings or published in medical journals.

What if you change your mind?

You can opt out of the study even after reading this information sheet and signing the consent form. You just need to tell your doctor.

Dr JM Walker: Consultant Cardiologist & Clinical Director, **Dr PMI Sutton**: Associate Director & Sen Lecturer, **Dr P Taggart**: Reader in Medicine, **Dr RH Swanton**: Consultant Cardiologist, **Professor J Betteridge**: Professor of Endocrinology & Metabolism, **Dr CCT Smith**: Sen Lecturer, **Dr M Mocanu**: Hon Lecturer, **Ms JK Walker**: Cardiac Rehabilitation, **Professor LH Opie**, Professor of Medicine & Director of Hatter Institute (UCT).

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2

INFORMED CONSENT FORM

Study Title: **An investigation into the effects of potential cardioprotective therapies on human heart muscle**

Name of Principal investigator: Professor Derek M Yellon

1. HAVE YOU RECEIVED THE INFORMATION SHEET ABOUT THIS STUDY? YES/NO

2. HAVE YOU HAD AN OPPORTUNITY TO ASK QUESTIONS AND DISCUSS THE STUDY? YES/NO

3. HAVE YOU RECEIVED SATISFACTORY ANSWERS TO YOUR QUESTIONS? YES/NO

4. HAVE YOU RECEIVED ENOUGH INFORMATIONJ ABOUT THE STUDY? YES/NO

5. DO YOU UNDERSTAND THAT YOU ARE FREE AT ANY TIME TO WITHDRAW FROM THE STUDY? YES/NO

6. DO YOU AGREE TO TAKE PART IN THIS STUDY? YES/NO

INVESTIGATOR'S DECLARATION

I have explained the above study to the patient

Signed _____ **Date**_____

PATIENT'S DECLARATION

I have had the nature and purpose of the study explained to me and agree to participate

Signed _____ **Date**_____

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SHORT COMMUNICATION

Exogenous SDF-1α Protects Human Myocardium from Hypoxia-Reoxygenation Injury via CXCR4

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Keywords SDF-1α · Stromal derived factor · CXCR4 · Ischaemia-reperfusion injury · Cardioprotection

Introduction

ST-segment elevation myocardial infarction (STEMI) is a consequence of atherosclerotic plaque rupture and thrombotic occlusion of the coronary artery causing downstream ischaemia and, eventually, cell death. The most effective therapeutic strategy for STEMI is timely reperfusion by primary percutaneous coronary intervention (PPCI). Such reperfusion is a prerequisite for myocardial salvage, leading to smaller infarct sizes and improved clinical outcomes [1, 2]. However, reperfusion can itself inflict further injury, termed ischaemia-reperfusion injury (IRI). Despite PPCI, a recent study found 30-day, 1-year, and 5-year mortality following STEMI to be 7.9 %, 11.4 %, and 23.3 %, respectively [3]. Consequently, novel strategies to mitigate the deleterious effects of IRI are paramount.

Stromal derived factor-1α (SDF-1α or CXCL12) is a chemokine that has demonstrated cardioprotective activity in mice [4]. We recently demonstrated that exogenous SDF-1α improved functional recovery of ex vivo rat cardiac papillary muscle subjected to hypoxia and reoxygenation (simulated IRI) [5]. This effect was abrogated by pre-treatment with AMD3100, a highly specific antagonist of the SDF-1α receptor, CXCR4.

However, it is not known whether SDF-1α can similarly protect human heart tissue and whether any such protection is afforded via CXCR4. We address this question using isolated human atrial trabeculae subjected to simulated IRI.

Methods

Human Atrial Trabeculae Hypoxia-Reoxygenation Experiments

The study received Local Research Ethics Committee approval and was carried out in accordance with the University College London Hospitals NHS Trust guidelines. A right atrial appendage sample was harvested from 47 patients with chronic stable angina undergoing cannulation for cardiopulmonary bypass for CABG. All patients were aged 18–80 years and there were no significant differences in their baseline characteristics **(see** Table 1**)**. Patients with diabetes, impaired renal or ventricular function, dilated left atria, unstable angina, or a history of arrhythmias or on rhythm stabilising medications were excluded.

Trabeculae were randomly allocated to [1] control ($n = 11$), [2] hypoxic preconditioning ($n = 10$), [3] SDF-1α pre-treatment ($n = 11$), [4] AMD3100 + SDF-1α pre-treatment ($n = 10$), and [5] AMD3100 pre-treatment ($n = 5$). Two separate trabeculae were collected for immunofluorescent staining. The sample was placed in ice-cold buffer prior to careful dissection of the trabeculae. Isolated trabeculae (≤1.2 mm in diameter and ≥2.0 mm in length) were suspended in a heated (37 °C) organ bath with one end connected to a force transducer. Samples were superfused with oxygenated modified Tyrode's buffer (95 % O_2/5 % CO_2) at 37 ± 0.5 °C and

A. Malik and D. I. Bromage contributed equally to this work and are joint first authors.

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Table 1 Patient baseline characteristics

	Control (n = 11)	Hypoxic preconditioning (n = 10)	SDF-1α (n = 11)	SDF-1α + AMD3100 (n = 10)	AMD3100 (n = 5)
Mean age (years)	61	64.8	64.6	63.4	61.6
Gender (male)	9 (82 %)	10 (100 %)	10 (91 %)	7 (70 %)	4 (80 %)
Good LV (>50 %)	11 (100 %)	10 (100 %)	11 (100 %)	10 (100 %)	5 (100 %)
eGFR >55 mL/min	11 (100 %)	10 (100 %)	11 (100 %)	10 (100 %)	5 (100 %)
Rhythm					
Sinus	11 (100 %)	10 (100 %)	11 (100 %)	10 (100 %)	5 (100 %)
Surgery					
CABG	7 (64 %)	4 (40 %)	7 (64 %)	5 (50 %)	1 (20 %)
AVR	4 (36 %)	5 (50 %)	0 (0 %)	5 (50 %)	4 (80 %)
CABG + AVR	0 (0 %)	1 (10 %)	4 (36 %)	0 (0 %)	0 (0 %)
Medications					
β-blocker	8 (73 %)	4 (40 %)	7 (64 %)	2 (20 %)	1 (20 %)
ACE inhibitor	5 (45 %)	5 (50 %)	5 (45 %)	3 (30 %)	1 (20 %)
Calcium channel blocker	2 (18 %)	0 (0 %)	1 (9 %)	1 (10 %)	1 (20 %)
Nitrate	2 (18 %)	2 (20 %)	1 (9 %)	1 (10 %)	1 (20 %)
Statin	8 (73 %)	2 (20 %)	7 (64 %)	5 (50 %)	3 (60 %)
MRA	1 (9 %)	1 (10 %)	0 (0 %)	0 (0 %)	0 (0 %)
Diuretic	2 (18 %)	1 (10 %)	1 (9 %)	0 (0 %)	0 (0 %)
Anti-arrhythmic	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)
Trabecular dimensions					
Length (mm)	4.31	5.16	5.05	3.86	4.6
Diameter (mm)	0.97	1.01	1.09	1.18	0.89

LV, left ventricle; eGFR, estimated glomerular filtration rate; CABG, coronary artery bypass graft surgery; AVR, aortic valve replacement; ACE, angiotensin converting enzmye; MRA, mineralocorticoid receptor antagonist

*Data expressed as number (%) or mean

pH 7.4 ± 0.5 [5]. The muscle was electrically paced at 1 Hz and stretched until the maximum force of contraction (the peak of the Frank-Starling curve) was achieved. The muscle was subsequently allowed to stabilise for 90 min before being subjected to 60 min of hypoxia by superfusion with equiosmolar, glucose-free hypoxic modified Tyrode's buffer (95 % N_2/5 % CO_2), pH 7.4 ± 0.5 and electrical stimulation at 3 Hz. The muscle was reoxygenated for 60 min with normoxic buffer and 1 Hz stimulation, to simulate reperfusion. Hypoxic preconditioning, consisting of 4.5 min hypoxia and pacing at 3 Hz followed by 6 min reoxygenation and pacing at 1 Hz, was applied immediately prior to the index hypoxic period as a positive cardioprotective control [5]. SDF-1α (25 ng/ml), AMD3100 (10 μg/ml) or saline vehicle were administered for 30 min and 40 min respectively prior to index hypoxia, concentrations that were based on previous publications [4, 5].

Immunohistochemistry

In a separate group of experiments, isolated human atrial trabeculae were frozen and mounted in OCT before being cut into 5 μm sections at −20°C in a microtome-cryostat and transferred to slides. Sections were fixed with HistoChoice (Sigma-Aldrich, UK) for 20 min at room temperature and washed with PBS, before blocking with 5 % BSA/PBS for 60 min. Immunofluorescent co-staining of CXCR4 and cardiomyocytes was performed using rabbit monoclonal anti-CXCR4 (ab124824) and mouse anti-cardiac troponin T (ab8295) from Abcam (Gillingham, UK). Anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 555 secondary antibodies were purchased from Abcam. Cardiomyocyte co-stained samples were incubated in anti-CXCR4 and anti-cardiac troponin T, diluted in 1 % BSA/PBS 1:100 and 1:10 respectively, overnight at 4°C. Following washing and incubation with the appropriate secondary antibody diluted 1:400 in the same buffer for 60 min at room temperature, samples were washed again and coverslips mounted using fluorescence mounting medium (Dako, Ely, UK). 0.1 μg/ml Hoechst 33,258 nuclear stain (Life Technologies, Paisley, UK) was added with the secondary antibodies to all sections. Preparation of control sections was identical and they were incubated either with 1 % BSA/PBS only (unstained control) or with the relevant secondary antibody in the absence of any primary antibody. After drying, Alexa 488 and Alexa 555 fluorescence was

imaged using a 40× oil immersion objective, by sequential scanning using the 488 nm and 543 nm lines of a Leica SP5 confocal microscope and collecting emitted light at 500–530 nm and 580–650 nm respectively. Control experiments were performed to confirm the absence of fluorescence bleed-through or non-specific staining with secondary antibodies alone.

Statistics

The final force of contraction in the human atrial trabeculae hypoxia-reoxygenation experiments was expressed as a percentage of baseline contractility to give recovery of function. Values are expressed as mean ± SEM. Comparisons between more than 2 groups were made using 1-way analysis of variance (ANOVA). Fisher's protected least significant difference post hoc test was used for between-group comparisons. Differences were considered statistically significant when $P < 0.05$.

Results

In control trabeculae, the mean recovery of function after hypoxia and reoxygenation was 27 ± 2 % [1]. Recovery of

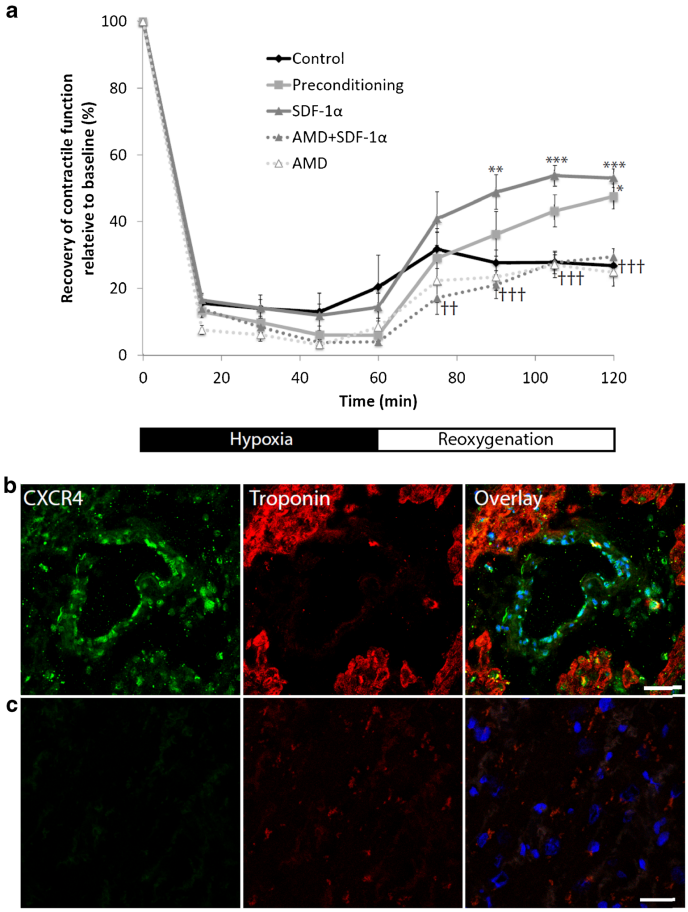

Fig. 1 The SDF-1α-CXCR4 axis protects human myocardium from hypoxia-reoxygenation injury: **a** Recovery of contractile function during simulated ischaemia and reperfusion applied to isolated human atrial trabeculae is improved by pre-treatment with exogenous SDF-1α to a level similar to that conferred by hypoxic preconditioning. N = 11 atrial trabeculae in the control and SDF-1α pre-treatment groups, 10 in the hypoxic preconditioning and AMD3100 + SDF-1α pre-treatment groups, and 5 in the AMD3100 pre-treatment group. *SDF-1α vs. control, †AMD + SDF-1α vs. SDF-1α (*$P < 0.05$, **$P < 0.005$, ***$P < 0.001$). **b**, Immunofluorescent staining of isolated human atrial trabeculae demonstrating the distribution of CXCR4 (green) in relation to cardiomyocytes (red). **c**, Immunofluorescent staining using secondary antibody in the absence of primary antibody. Representative images from N = 2 independent experiments, 50 μm scale bar

function in samples pre-treated with SDF-1α was significantly increased (53 ± 3 %, $P < 0.05$ vs. control, Fig. 1), which was similar to that in control trabeculae subjected to hypoxic preconditioning (48 ± 4 %, $P < 0.05$ vs. control).

The role of signalling via CXCR4 was investigated by pretreating trabeculae with the specific CXCR4 inhibitor, AMD3100, which abrogated the protective effect of SDF-1 (30 ± 2 %, $P < 0.05$ vs. SDF-1α alone). AMD3100 alone had no effect on functional recovery (25 ± 4 %). Furthermore, immunofluorescent staining confirmed the presence of CXCR4 receptors on cardiomyocytes in human atrial trabeculae (Fig. 1).

Discussion

SDF-1α is a small CXC chemokine that is expressed by several organs and tissues, including endothelial cells and cardiomyocytes in the heart [6]. Its cognate receptors CXCR4 and CXCR7 are similarly present in a range of tissues, including cardiomyocytes. The SDF-1α/CXCR4 axis has garnered considerable interest due to its role in stem cell homing, angiogenesis and ventricular remodelling after myocardial infarction, and has been used to target stem cells to ischaemic tissue, thereby improving LV dimensions and function [7].

We and others have previously demonstrated a cardioprotective role for SDF-1α on both infarct size in isolated perfused rat hearts and recovery of function in isolated rat papillary muscle [5, 8]. Both of these beneficial effects were abolished by AMD3100, all of which evidences a cardioprotective role for the SDF-1α/CXCR4 axis. Elsewhere, the application of exogenous SDF-1 has been shown to reduce apoptosis in isolated cardiomyocytes and SDF-1 infused into the murine LV cavity in vivo produced significantly smaller infarct sizes after IRI, both effects that were abrogated by AMD3100 [9].

This ex vivo functional model of hypoxia and reoxygenation translates the aforementioned findings to human myocardium for the first time, in the native milieu of the functional heart. This study confirms [1] the ability of exogenous SDF-1α to protect human atrial trabeculae from the detrimental effects of simulated IRI; [2] the role of CXCR4 in this mechanism, as evidenced by attenuating the benefits of SDF-1α using the specific receptor blocker AMD3100; and [3] the presence and distribution of CXCR4 in human adult cardiomyocytes and endothelial cells.

There is evidence that the cardioprotective utility of SDF-1α/CXCR4 may be conferred by activating intracellular prosurvival kinases, although the specific mechanism remains unknown [5]. However, it is thought to converge on the mitochondrial permeability transition pore (mPTP) and effect protection by delaying its opening and consequent necrotic cell death [10]. The exact mechanism of protection afforded by SDF-1α/CXCR4 should be the focus of further investigation in both basic and clinical studies.

In summary, our findings support the hypothesis that exogenous SDF-1α is a preconditioning mimetic, herein shown to exert acute protection against the deleterious effects of hypoxia and reoxygenation and that this protection, in the isolated human atrial trabeculae, occurs via the CXCR4 receptor.

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ischemia (MI) and ischemia-reperfusion. The mitochondrial enzyme thiosulfate sulfurtransferase (TST) has a putative role in removal of H_2S and inhibition may provide an alternative means of therapeutically increasing H_2S bioavailability. This study investigated the hypothesis that deletion of TST in mice (*Tst* $^{-/-}$) would reduce infarct size and improve outcome following MI.

Methods TST expression was assessed by qRT-PCR and Western blotting. *Tst* $^{-/-}$ and wild-type (WT) mice were anaesthetised for assessment of cardiac structure and function by high frequency ultrasound and for measurement of blood pressure. MI was induced by coronary artery ligation *in vivo* , or *ex vivo* in perfused hearts, when 30 mins ischemia was followed by 120 min reperfusion.

Results qRT-PCR and Western blots confirmed the presence of TST in the murine heart and also deletion in *Tst* $^{-/-}$. *Tst* $^{-/-}$ mice survived to adulthood, had no change in blood pressure relative to WT and and had normal cardiac structure and function. Expression of the H_2S synthesising enzyme, cystathionine gamma-lyase (CSE), was reduced in Tst$^{-/-}$ relative to WT hearts ($n = 5$, $p < 0.01$). Following MI the incidence of cardiac rupture was increased in Tst$^{-/-}$ mice (66%, vs 20% in WT). Studies in isolated hearts revealed increased infarct size in Tst$^{-/-}$ ($88 \pm 2\%$ area at risk, vs $65 \pm 6\%$ in WT, $n = 6$, $p = 0.01$).

Discussion TST is present in the heart and deletion does not influence structure or function. *Tst* $^{-/-}$ mice have reduced cardiac CSE expression, suggesting reduced H_2S synthetic capacity. This may underlie the increased susceptibility of *Tst* $^{-/-}$ mice to myocardial injury and increased mortality.

Conclusion TST may act with CSE to regulate H_2S availability. Alteration in the balance of these enzymes has no overt physiological effect, but is associated with reduced capacity of the heart to resist ischaemic stress.

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222 SRC-DEPENDENT TYROSINE PHOSPHORYLATION OF HIC-5 REGULATES HIC-5 SUBCELLULAR LOCALISATION AND ACTIN CYTOSKELETON DYNAMICS IN RAT VASCULAR SMOOTH MUSCLE CELLS IN RESPONSE TO NORADRENALINE AND ENDOTHELIN-1

This abstract has been withdrawn.

223 A NOVEL IMMUNOGLOBULIN G AUTOANTIBODY AGAINST LOW DENSITY LIPOPROTEIN (LDL) WITH PATHOGENIC FUNCTIONS

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Introduction IgG autoantibodies reacting with different forms of modified LDL are found in blood and have been associated with cardiovascular events related to atherosclerosis. However there is a controversy as to whether such antibodies are pathogenic. We set out to generate monoclonal autoantibodies reacting with LDL for further mechanistic studies.

Methods/Results We fused splenocytes from a LDL receptor deficient (*Ldlr*$^{-/-}$) atherosclerotic mouse with the Sp2/0 myeloma cell line, and screened hybridoma culture supernatants by ELISA for reactivity with solid-phase LDL. We selected a monoclonal IgG2b antibody, designated LO9. Sequencing of the LO9 variable heavy (V_H) and variable light (V_L) regions revealed numerous somatic mutations in the complementarity determining regions (CDR) of both the V_H and V_L. Together with the IgG2b subclass, these indicate that LO9 is not a natural antibody but has developed from an adaptive immune response. To investigate interaction of LO9 with LDL we performed *in vitro* binding assays. LO9 reacted with LDL directly adherent to ELISA plates, but not with LDL immobilised by polyclonal anti-apoB. Less reactivity was noted with Cu^{++} oxidised-LDL and malondialdehyde-conjugated LDL (MDA-LDL). LO9 binding to adherent LDL was not inhibited by fluid-phase LDL in excess and the LO9 epitope was not abolished by organic solvents, suggesting that it is not lipid. In functional assays, addition of macrophages to LO9 bound to adherent LDL *in vitro* led to significant TNF release, an effect that was blocked by inhibition of Fc gamma receptors (FcγRs), and which was not seen when macrophages were incubated with adherent LDL alone. Expression of the LO9 epitope on adherent LDL was greatly increased by incubation with conditioned medium (CM) from activated macrophages, consistent with the LDL epitope being further revealed by the action of macrophage-derived factors. Expression of the LO9 epitope in atherosclerosis was confirmed by immunohistochemical staining of human and mouse lesions.

Following intra-venous injection of LO9 labelled with a near infra-red dye (LO9–750), fluorescence molecular tomography imaging demonstrated specific localisation to a region of interest covering the aortic arch of *Ldlr*$^{-/-}$ mice but not wild type animals. Confocal microscopy of extracted aortae *en face* confirmed that LO9 localised in atherosclerotic regions beneath endothelium and in the vicinity of macrophages.

Conclusions We believe LO9 is the first example of an autoantibody that reacts with an allosteric epitope on LDL, revealed by adhesion to a surface and/or by the action of factors derived from activated macrophages. Binding of LO9-like antibodies to LDL trapped on matrix in the arterial wall could lead to promotion of atherosclerosis via ligation of macrophage FcgRs and release of TNF and other proinflammatory mediators.

224 STROMAL DERIVED FACTOR 1 ALPHA IS A MEDIATOR OF CONDITIONING IN HUMAN AND RAT MYOCARDIUM

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Background We have recently demonstrated that stromal cell-derived factor 1α (SDF-1α) limits myocardial infarct size (IS) in a murine model of ischaemia-reperfusion injury (IRI) via its receptor CXCR4. This study aimed to investigate the role of SDF-1α in the cardioprotection conferred by remote ischaemic conditioning (RIC) using three different human and animal models.

Methods *1) In vivo* RIC: Rats were anaesthetised with sodium pentobarbitone (60mg/kg i.p.), intubated and ventilated. Animals were randomly allocated to receive intravenously either a specific inhibitor of CXCR4, AMD3100 (10μg/ml), or vehicle (0.9% saline). Animals then underwent either a RIC protocol (3 × 5 min hind limb ischaemia) or a sham procedure for the same time period. Complete cessation of blood flow in the RIC group was confirmed by Doppler measurement. All groups were subjected to 30 min left coronary artery occlusion and 2h reperfusion (IR), after which the heart was removed and analysed for IS as a proportion of area at risk (IS/AAR%). *2) Ex vivo* RIC: Coronary effluent was collected from isolated perfused rat hearts following either stabilisation (C_{eff}) or during a preconditioning protocol (3 × 5 min, IPC_{eff}). Recipient hearts were perfused with effluent for 10 min (± 5 μg/ml AMD) prior to 35 min LCA occlusion and 60 min reperfusion. Infarct size was measured as above. *3) Isolated human*